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Gene and protein expression as potential biomarkers for differentiating ovarian carcinoma from malignant mesothelioma

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ABBREVIATIONS

°C	Degrees celsius
ABC	ATP-binding cassette
B72.3	Tumor associated glycoprotein
bFGF	Fibroblast growth factor 2 (basic)
bp	base pairs
BRCA1/2	Breast cancer gene 1/2
CA	California
CA125	Cancer antigen 125
CD15	Carbohydrate adhesion molecule
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridization
cm	centimeters
COX-2	Cyclooxygenase-2
CT	Computed tomography
DMPM	Diffuse malignant peritoneal mesothelioma
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBS	Ets binding site
EGFR	Epidermal growth factor receptor
EHF	Est homolugous factor
EMA	Epithelial membrane antigen
FC	Flow cytometry
FIGO	International federation of gynecology and obstetrics
g	grams
GAP	GTPase-activating protein
GDI	GDP-dissociation inhibitor
GDP	Guanosine diphosphate
GEF	GDP/GTP exchange factor
GEP	Progranulin-epithelin
GTP	Guanosine triphosphate
GUS	beta-glucuronidase
H&E	Hemotoxylin and eosin
Her-2	Human epithelial growth factor receptor 2
HLA-G	Human leukocyte antigen G
IHC	Immunohistochemistry
IL-8	Interleukin 8
MM	Malignant mesothelioma
mM	milli molar
mm	millimeter
MAPK	Mitogen-activated protein kinase
mg	milligram
min	minutes
ml	millilitre
MMP 2/9	Matrix metalloproteinase 2/9
MMR	Mismatch repair
mRNA	messenger RNA
NCBI	National centre of biotechnology information
ng	nanogram
nm	nanometer
NSOC	National survey of ovarian cancer
NTC	Non template control
OC	Ovarian carcinoma
OS	Overall survival
p53	Tumor protein 53
$p75^{NGFR}$	Nerve growth factor receptor
PBS	Phosphate buffered saline
PCI	Peritoneal cancer index
PCR	Polymerase chain reaction
PFS	Progression free survival
Pi	Inorganic phospate
PNT	Pointed domain
PPC	Primary peritoneal cancer
PRAME	Preferentially expressed antigen of melanoma
qRT-PCR	Quantitative real-time PCR
RA	Retinoic acid
RAR	Retinoic acid signaling
RAS	Rat sarcoma
RBEL-1	Rab like protein 1
REP	Rab escort protein
RM	Reactive mesothelial cells
RNA	Ribonucleic acid
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
sec	second
Sip1	Smad-interacting protein 1
SNP	Single nucleotide polymorphism
SV40	Simian virus 40
TGM	Tumor-grade-metastasis
TIMP-2	Tissue inhibitor of matrix metalloproteinase 2
Tm	Melting temperature
TN	Tumor necrosis
TNM	Tumor-node-metastasis
TP53	Tumor protein 53
TrkA	Neurotrophic tyrosine kinase receptor, type 1
TTC	Transitional cell carcinoma
TTF-1	Thyroid transcription factor-1
TVS	Transvaginal ultrasonography
ul	microlitre
V	Volt
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WT1	Wilms' tumor-1
XIAP	X-linked inhibitor of apoptosis

ABSTRACT

Ovarian carcinoma (OC), primary peritoneal carcinoma (PPC) and malignant mesothelioma (MM) are all highly aggressive tumors that share common morphological features in addition to being histogenetically related. It is of great interest to investigate the underlying molecular differences amongst these tumor types. The identification of differential gene expression signatures has the potential to serve as biomarkers, which can be used for diagnosis and prognosis of these tumors. This study examines the gene expression levels of *RAB25, PRAME* and *EHF*, in addition to RAB25 protein expression levels, in large sample material consisting of primary OC, OC/PPC effusions and MM effusions. The results showed higher expression of *RAB25, PRAME* and *EHF* mRNA in OC/PPC effusions compared to MM effusions, in addition to significantly higher expression of *EHF* mRNA primary OC compared to OC/PPC effusions. The study also revealed pronounced differences in RAB25 protein expression between OC/PPC effusions and MM effusions, along with significantly higher RAB25 protein expression in histological grade 3 compared to grade 1-2 within the OC/PPC effusions. Another result generated in this study was that there is a significantly higher expression of *EHF* mRNA in the pre-chemotherapy OC/PPC effusions, and this upregulation proved to be correlated with poor progression free survival survival, also independent in COX multivariate analysis.

1. INTRODUCTION

1.1. Cancer

Cancer is the leading cause of death worldwide. The disease was responsible for 7.4 million deaths globally in 2004 and accounts for approximately 13% of all deaths worldwide (1). There were 26,000 new cases of cancer in Norway in 2008, and the Cancer Registry of Norway estimates that one out of three Norwegians will be diagnosed with cancer before the age of 75 years (2).

The underlying causes of cancer are abnormalities in DNA sequence, copy number, rearrangements, or expression. The accumulation of multiple genetic changes can lead to essential alterations in cell physiology which have the potential of enabeling the cell to escape from normal controls on cell growth and proliferation. This lack of control on cellular processes can result in sustained angiogenesis, invasion and metastasis which further can lead into the development of a clinically evident tumor (3).

1.2. Ovarian carcinoma

1.2.1. Epidemiology

Cancer of the ovary ranks as the second most common gynecological cancer after uterine cancer with an estimated 21,800 newly diagnosed cases in the United States for 2010. Ovarian carcinoma (OC) has the highest death toll among the gynecologic cancers with more than 50% of the diagnosed women dying from

this disease (4). Similar numbers are observed in worldwide statistics for 2008, with 225,000 new cases diagnosed and 140,000 deaths (5). The highest incidence of OC is found women in Europe and North America, a somewhat lower incidence is found in women from Central and South America, whilst the lowest incidence is observed among women in Africa (6). Comparing the age-standardized incidence rates for the Nordic countries in 1995, the numbers show that Sweden has the highest incidence with an age-standardized rate of 20.5 per 100,000, whilst Finland has the lowest with a rate of 15. Denmark, Norway and Iceland have age-standardized rates of 19.5, 18.6 and 17.8 respectively (7). More recent data show that Norway had 457 new cases of OC in 2008, and that there were 315 deaths caused by the malignancy in 2007 (2).

OC is known to be a disease of older women with the median age of patients being 60 years (8). Incidence rates increase with each decade of life and come to a peak around 75-80 years of age. Considering non-hereditary forms of the disease it is uncommon to develop OC before the age of 40 years. Hereditary predisposition for the disease is linked to approximately 5-10% of the cases, and the average lifetime risk of OC for women in developed countries is one in 70 (8, 9).

1.2.2. Histopathology

There is great histological heterogeneity among ovarian tumors, and primary classification is based upon the histogenesis of the normal ovary. The majority of

ovarian cancers are classified as epithelial (OC) and they make up 80-90% of all cases. The nonepithelial ovarian cancers consist of germ cell tumors and sex-cord stromal cell tumors, accounting for approximately 15- 20% and 5-10% respectively (10-12). OC originates from malignant transformation of the coelomic epithelium which covers the surface of the ovaries (13, 10). The coelemic epithelium is multipotential and can differentiate into various types of epithelium such as mullerian, endometrial, endocervical, tubal and intestinal types thus giving rise to the wide variety of epithelial tumors observed. Tumors are consequently histologically classified as serous, mucinous, endometrioid, clear cell, mixed epithelial, undifferentiated, transitional (Brenner) and squamous cell. The degree of cellular proliferation, nuclear atypia and the presence of stromal invasion are the criteria by which ovarian tumors are classified as benign (cystadenoma), borderline or malignant (carcinoma) (9, 10, 12).

1.2.3. Etiology

The molecular events underlying the development of OC are poorly understood. However, it is evident that OC is a multifactorial disease, with genetic, environmental, and endocrinological factors directly or indirectly related to its carcinogenesis (11, 14). Age is considered a major risk factor, as OC primarily is a disease of older women. Other risk factors are nulliparity, early menarche and late menopause. These risk factors suggest that a prolonged nulliparous state with uninterrupted ovulation is capable of causing repeated stimulation of the ovarian surface epithelium, and that this repeated stimulation is causative of

malignant transformation (8, 11, 13-16). Additional risk factors are obesity due to its relationship with sex steroids, and chronic inflammation due to its alteration of the ovarian microenvironment (15). The genetic risk factors of Hereditary Breast and Ovarian cancer and Hereditary Nonpolyposis Colorectal cancer (Lynch Syndrome) are also associated to OC due to their associated mutations in oncosupressor genes BRCA1 and BRCA2, and the DNA mismatch repair (MMR) genes respectively. Protective factors for OC include multiparity, oral contraceptive use, and surgical risk reduction with tubal ligation or hysterectomy (8, 11, 13-16).

1.2.4. Grading and staging

Histopathological tumor grading where the degree of differentiation of the tumor cells is measured microscopically can differentiate carcinomas. There is a general tumor grading system applicable to most carcinomas which states that grade 1 tumors are well differentiated and are frequently associated with good prognosis, grade 2 tumors are moderately well differentiated and have an intermediate prognosis whilst grade 3 are poorly differentiated and often associated with an unfavorable outcome (17). In the case of OC there are several systems used to grade the neoplasms with the two most commonly used throughout the world being that of the International Federation of Gynecology and Obstetrics (FIGO) and that of the World Health Organization (WHO) (18). The FIGO system is based on architectural features, and it utilizes three grades (1-3). The grades depend upon the proportion of glandular or papillary structures relative to areas of solid tumor growth within a specific tumor. Grades

1, 2 and 3 correspond to <5%, 5-50%, and >50% solid growth respectively (18-19). In the WHO system the grade is based on both architectural and cytological features, but this is not done in a quantitative manner (18). As a result from the change in the perception of OC, from it being considered to be a single disease to the recognition of the heterogeneity of this disease, there has emerged a new system for grading of OC, which is based on a histotype-specific approach. This system uses grading parameters that are individualized for the different histological types (20). Another system for OC classification is based on dividing tumors into two groups; type I and type II. The division is based on the diverse nature of the tumors and segregates them according to clinical, pathological and molecular features (21).

Staging of OC is mainly done surgically in order to determine the extent of disease. The FIGO system developed in 1988 (shown in Table 1) is most commonly used worldwide to stage OC (18, 22).

Table 1. Ovarian carcinoma: FIGO staging nomenclature (1988) (Ref 22)

Stage I	**Growth limited to the ovaries.**
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact.
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact.
Ic[a]	Tumor either Stage Ia or Ib, but with tumor on surfcae of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
Stage II	**Growth involving one or both ovaries with pelvic extension.**
IIa	Extension and/or metastases to the uterus and/or tubes.
IIb	Extension to other pelvic tissues.
IIc	Tumor either stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
Stage III	**Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equal Stage III. Tumor is limited to the true pelvis, but with the histologically proven malignant extension to small bowen or omentum.**
IIIa	Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentry.
IIIb	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative.
IIIc	Peritoneal metastasis beyond the pelvis >2cm in diameter and/or positive retroperitoneal or inguial nodes
Stage IV	**Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV.**

[a] In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or acites.

1.2.5. Clinical presentation, diagnosis and screening

OC patients often present with non-specific symptoms which make early diagnosis difficult. Early-stage patients can present with symptoms such as irregular menses and urinary frequency, constipation and dyspareunia due to tumor growth and occasionally lower abdominal distension, pressure and pain (9, 10, 23). In advanced disease when there is tumor spread throughout the abdominal cavity the symptoms are often related to the ascites, omental or bowel involvement, and include abdominal distension, bloating, constipation, nausea, anorexia and early satiety. Patients can also present with a shortness of breath due to pleural effusions (8-10, 13, 16).

In order to diagnose the disease, a key factor is to detect the presence of a pelvic mass on rectovaginal examination as a solid, irregular, fixed pelvis mass is a highly suggestive indication of an ovarian malignancy. In order to obtain a certain diagnosis an exploratory laparotomy is required (9, 16). In general two thirds of OC are at an advanced stage upon diagnosis (10).

Screening for OC is currently conducted via the use of transvaginal ultrasonography, measurement serum CA-125 values, and rectovaginal pelvic examinations (11). Due to the relatively low prevalence of OC, strategies for early detection should have high sensitivity (>75%) and very high specificity (99.6%) in order to attain a positive predictive value of 10% or greater. Serum CA-125 concentration does not have the sensitivity or specificity to function

alone in screening. Greater specificity can be achieved by combination of CA-125 measurement and transvaginal ultrasonography (TVS), by monitoring CA-125 concentration over time, or both (13, 23). However these screening modalities are at present unable to detect early-stage disease and lack the recommended sensitivity. As such they are discouraged to be used in the general population, and have a limited relevance for use in a high risk population (24). Recently there have been advances within the field of proteomics. These advances have lead to the development of new technologies that may be able to identify new potential biomarkers that are present in small amounts in serum. The novel candidate biomarkers are thought to improve the level of sensitivity and provide a more effective means for screening (23, 25).

1.2.6. Current therapy

Surgery is an important part of ovarian cancer treatment. In early-stage disease it is pivotal to perform thorough staging in order to define the correct extent of malignancy. Exploratory laparotomy is the method of choice to obtain histological confirmation, staging and tumor debulking. In addition to tumor debulking, early-stage disease patients should also be subjected to peritoneal washing, peritoneal biopsies representative of the entire abdominal cavity, and a retroperitoneal assessment that involves both the pelvic and para-aortic area. These procedures are important in order to determine whether there is a spread of the disease, and if so to what extent (16). Studies have revealed that approximately one third of the assumed early-stage patients undergoing initial surgery prove to be understaged, and present with advanced-stage disease with

metastatic spread (9, 13, 16). Advanced-stage disease patients are also subjected to surgery as the tumor debulking procedure is an essential part of the initial treatment. It is clearly demonstrated that having a residual tumor 1 cm or less in diameter is associated with an increased survival rate for the patient (8, 16). A specialist gynecological oncologist at a high-volume center should perform tumor debulking surgeries of advanced-stage patients, as studies have shown that this will give a 5-8 months median survival benefit for these patients (13).

Postoperative adjuvant chemotherapy is required for most OC patients in order to attempt to eradicate any residual disease (8). Today's gold standard for primary chemotherapy treatment is the carboplatin-paclitaxel regimen (26). Carboplatin is a platinum analogue, its mode of cytotoxic action is mediated through the interaction with DNA to form DNA adducts. These DNA adducts are primarily intrastrand crosslink adducts and they are responsible for activating several signal transduction pathways such as those involving ATR, p53, p73, and MAPK. The eventual result of this activation is the goal of apoptosis. Carboplatin is the most commonly used platinum analogue used for treating OC, as it has equal activity but considerably less toxicity than other platinum compounds such as cisplatin (27-28). Paclitaxel is the taxane component of adjuvant therapy for OC and it works through promoting the polymerization of tubulin, thereby causing cell death by disrupting the normal microtubule dynamics required for cell division and vital interphase processes (29).

1.2.7. Clinicopathologic and molecular prognostic factors

The most significant prognostic factor is stage of disease at diagnosis (30). This is evidence-based on a study carried out by the National Survey of Ovarian Cancer (NSOC). The study reports of five-year survival for patients with FIGO stage Ia, Ib and Ic disease of 92%, 85% and 82% respectively. The five-year survival was 67% in stage IIa disease, 56% in stage IIb, and 51% in stage IIc. The worst five-year survival was manifest in stage III and IV disease with 39% for stage IIIa, 26% for stage IIIb, 17% for stage IIIc and 12% for stage IV (31). Histologic type is also a prognostic factor with clear cell and small cell carcinomas being associated with a worse prognosis than the other histological types. Tumor grade is another prognostic factor as five-year survival rates of patients with invasive cancers of early stage (I and II) vs advanced stage (III and IV) are; 87% and 38% respectively for grade 1 disease; 70% and 25% respectively for grade 2 disease, and 64% and 19% respectively for grade 3 disease (30). An additional prognostic factor is DNA ploidy, with diploid tumors being associated with a better survival than aneuploid tumors in both early- and advanced-stage disease (32). The volume of residual disease left after primary cytoreductive surgery is also indicative of survival outcome, with optimal debulking, indicating a residual tumor of 1 cm or less, being associated with a significant survival advantage (33-34).

Several molecular biomarkers are predicted to have an impact on response and outcome of OC, and among them is the large membrane-bound glycoprotein CA 125. Circulating level of CA 125 is associated with degree of tumor burden and as

such it can be used to monitor disease at presentation and during progression (35-36). P-glycoprotein and other ABC transporters can affect intracellular drug concentrations and *in vitro* studies have linked their overexpression to chemosensitivity of ovarian cancer cells to anthracyclines and paclitaxel (35, 37). Another biomarker in OC is vascular endothelial growth factor (VEGF), due to the fact that its presence in high amounts in preoperative serum samples is associated with poor survival. There is an 8-fold risk of cancer-related death if a patient present with serum VEGF ≥389 pg/ml, compared to patients with lower antigen values (38). Worsened prognosis of OC was also found to be linked to overexpression of EGFR and HER-2, tyrosine kinase receptors belonging to the epidermal growth factor family that activate pro-survival and -proliferation signal transduction pathways (39-40). Mutations of the tumor suppressor gene *TP53*, coding for p53 protein, have frequently been found in epithelial OC of all stages. As a result of the significantly higher frequency of over-expression of p53 in advanced-stage III/IV disease (50-60%) compared to stage I cases (10-20%), its expression levels can be used as a prognostic factor (41). In a similar manner as *TP53* mutations, mutations in *BRCA1* and *BRCA2* are also associated with poorer prognosis for OC patients (42). Actinin-4, cancer-associated molecules of the integrin, MMP and the Ets family are additional molecular factors that are associated with prognosis in OC (43-44). The biomarkers mentioned here represent a small selection out of the numerous biomarkers that are known to be associated with prognosis in OC, and new biomarkers are continuously being discovered (45-48).

1.3. Malignant mesothelioma

1.3.1. Epidemiology

Malignant mesothelioma (MM) used to be considered a rare form of cancer. Today's trends show that MM incidences are on the rise worldwide, and that these increases will continue until they reach their peak in 10 to 20 years time (49-51). Currently there are 2,000-3,000 new cases in the US each year, and the majority of cases occur in the age group 50-70 years (52-53). Comparing incidence rates globally, the observation is that among the United States, Europe, Japan and Australia the highest incidence is found in Australia, whilst the lowest incidence is found in Japan (50, 54). Statistics show an incidence of 15, 18, 7 and 40 cases/million population and peak years of 2004, 2015-2020, 2025 and 2015 for the Unites States, Europe, Japan and Australia, respectively (50). There is a clear difference between genders with MM incidences being over five times higher in men than in women (50, 52, 55-56). Incidence trends of MM in Norway comply with the worldwide incidence data for Europe and show that the majority of cases occur in men with an age-adjusted incidence rate of 16.6 per million compared to 2.3 among women (50, 57). Estimations stipulated this year; 2010, to be the incidence peak of MM in the Norwegian population (57).

Along with increased incidence rates, it is estimated that MM will cause over 10.000 deaths annually on a worldwide basis up until at least 2020 (58). Estimates have even been as high as 15,000- 20,000 deaths annually worldwide, placing MM in a category together with the most common male cancers

especially in Europe and Australia (50, 59). In Norway there were a total of 374 deaths related to MM in the time period 2004-2008 (2).

1.3.2. Histopathology

MM is an aggressive tumor that originates from mesothelial cells of serosal surfaces. The pleura is the most common site of origin with 65-70% of all cases, followed by the peritoneum with 30%, and the tunica vaginalis testis and pericardium with 1-2%. MM can arise from both the visceral and parietal peritoneum (60). In regards to the topic for this project, the prime focus is on diffuse malignant peritoneal mesothelioma (DMPM), although pleural tumors are additionally studied.

MM have three different histological types: epithelioid, sarcomatoid (or fibrous), and biphasic (or mixed) (49, 59-60). MM are most commonly of the epithelioid type (50%), with the remaining cases being sarcomatoid (10%) or mixed (40%; ref 59). In addition there are some rare variants of MM that share some characteristics with the epithelioid type. These include benign adenomatiod tumor and the borderline tumors such as well-differentiated papillary mesothelioma and multicystic mesothelioma (61). DMPM differs from its pleural counterpart, as the majority of tumors present with purely or predominantly epithelioid morphological features (62).

The epithelioid type of MM can grow in four different patterns: tubular, papillary, diffuse and deciduoid, of which papillary MM is the most common and often coexists with other patterns such as the tubular (53). A microarray-based study has shown that the epithelioid and sarcomatoid types of MM can be differentiated through their distinctive gene expression signatures and molecular characteristics (63).

1.3.3. Etiology

There is an undisputable link between asbestos exposure and the development of MM. Approximately 80% of MM are associated with asbestos exposure, and about 5% of asbestos workers develop this cancer (64). The long and thin asbestos fibers have long biopersistance and are able to penetrate the lung and confer malignancy by inducing a chronic inflammatory process that causes the mesothelial cells to undergo repeated cycles of damage and repair (52-53). Regarding DMPM, there is a theory that the asbestos fibers reach the peritoneal surface via coughing up from the lungs followed by swallowing.

Even though asbestos is known to be the primary carcinogen linked to the development of MM other key factors may also play a role. There is strong evidence linking radiation to the development of MM. Several cases report of MM in patients that have received radiation to the thorax or abdomen, or who have received the contrast agent Thorotrast intravascularly (52, 64-65). There is also a clear link between the development of MM and the combination of

genetics and environment. In the Turkish area of Cappadocia there has been observed a MM epidemic caused by erionite exposure in genetically predisposed individuals (66). In addition there are studies showing that the DNA virus Simian Virus 40 (SV40) is a cofactor in the causation of MM. The SV40 virus is a potent oncogenic virus that is capable of blocking tumor-suppressor genes as well as altering the karyotype and stability of the host genome by inducing structural and numerical chromosomal alterations (67). The SV40 virus is endogenous to the rhesus monkey, but the virus is thought to have been transmitted to humans through its presence in the poliomyelitis vaccines 40 to 50 years ago (68). However this is a controversial issue, as there has been found contradictory evidence to SV40 being a causative agent of MM. This evidence is based upon the fact that there is a high risk of false-positive PCR results owing to the presence of SV40 sequences in common laboratory plasmids (69).

1.3.4. Grading and staging

The International Mesothelioma Interest Group has published a TNM (tumor-node-metastasis) system that is used to predict prognosis (70). This system is designed for pleural MM, and does not specifically apply to DMPM. However, in 2001 the TGM (tumor-grade-metastasis) staging system emerged as a proposed staging system for peritoneal mesothelioma (71).

Table 2. TGM staging system. Peritoneal Mesothelioma

Primary tumor extent			
T1		PCI assessment between 0 and 28	
T2		PCI assessment between 28 and 39	
Mesothelioma histopathological grade			
G1		Multicystic, adenomatiod and well-differentiated papillary	
G2		Epithelial (epitheliod and tubopapillary)	
G3		Sarcomatous, biphasic and undifferentiated	
Metastasis			
Mx		Presence of metastasis can not be assessed	
M0		No evidence of metastasis	
M1		Lymphatic or parenchymal distant metastasis	
Stage system grouping		**Survival**	
		Median	**Estimated 3-year**
I	Any T, G1, M0	52.0 months	90-100%
II	T1, G2, M0 T1, G3, M0	32.5 months	60-70%
III	T2, G2, M0 T2, G3, M0	15.5 months	30-40%
IV	Any T, any G, M1	13.5 months	<20%
PCI = Peritoneal Cancer Index			

1.3.5. Clinical presentation and diagnosis

DMPM patients usually present with progressively severe non-specific abdominal pain, distension due to ascites, and occasionally organ impairment, such as bowel obstruction (49-50). The unspecific features of weight loss, fatigue, fever and night sweats, thrombocytosis and anemia have a tendency to develop later in the course of the disease. Due to the anatomical positioning of the peritoneum, the tumor is able to develop unnoticed within the body cavity resulting in a situation where the patient can have quite extensive tumor dissemination within the peritoneum by the time of diagnosis (50).

Accurate diagnosis of MM is important, not only for clinical management but also for legal matters in regards to compensation issues. Median survival is 12 months from diagnosis, and likelihood of survival beyond a year is less than 50% (50). Imaging through the use of computed tomography (CT) is useful for the detection, characterization, staging and guiding biopsy of peritoneal masses, but it is not sufficient as a means for establishing a diagnosis. An accurate diagnosis of DMPM requires the use of histological and cytological samples, thus biopsy by laparotomy, peritoneoscopy or effusion sampling is necessary. Immunohistochemisty (IHC) is the most widely-used ancillary technique for both the cytological and the histological specimens. In the case of equivocal IHC results, electron microscopy can be employed in order to obtain a final diagnosis (50, 60, 72). In addition there have also emerged DNA microarray based strategies for mesothelioma diagnosis (50).

In order to obtain an accurate diagnosis of MM by the use of IHC, cytological markers such as calretinin and Wilms' tumor 1 antigen (WT1) are employed to determine if the tissue is of mesothelial origin. Both epithelial membrane antigen (EMA) and desmin are used as markers to determine whether the tissue is malignant or not (60, 73). Another cytological marker is cytokeratin. Cytokeratin stains are important for confirming invasion and distinguishing MM from sarcomas and melanoma. MM must also be distinguished from adenocarcinomas, and the adenocarcinomas markers CEA, CD15, TTF-1 and B72.3 are used for this purpose as they are almost never expressed in MM (50).

1.3.6. Current therapy

The best treatment results have emerged from specialized centers using a combination of tumor debulking and intraoperative chemotherapy (74). Established protocols refer to cytoreductive surgery in order to remove as much tumor as possible. Results of surgery can vary from cases where there is incomplete resection, to cases where a peritonectomy has lead to the removal of complete parts of the peritoneum (60, 75).

No chemotherapy regimen has proven to be curative for MM; however chemotherapy plays an important role in palliation (50). Chemotherapy can be administered systemically or directly into the abdomen. Various combinations of cisplatin, irinotecan, cyclophosphamide, doxorubicin, dacarbazide, gemcitabine and pemetrexed are used for systemic chemotherapy. Perioperative

intraperitoneal chemotherapy uses a combination of two or three of the antineoplastic agents cisplatin, mitomycin C, fluoroacil, doxorubicin, and paclitaxel. The perfusate is preheated to 42.5°C before it is administered into the abdomen. Heating the perfusate leads to a synergistic effect with the chemotherapy agent which gives an enhanced toxicity. The direct exposure of antitumor agent to the peritoneal surface results in greatly enhanced drug concentrations and decreased systemic toxicity, and as such it is considered to be most effective against DMPM (53, 60).

1.3.7. Clinicopathologic prognostic markers

The most common clinical prognostic factors identified are histological type, performance status, gender, weight loss, chest pain, leukocyte count and clinical stage (76-77). Validated poor prognostic factors in MM include non-epithelial cell type, poor performance status, male sex, high white blood cell count and low hemoglobin levels (77-78).

Novel candidate indicators of prognosis include fibroblast growth factor receptor, the putative angiogenesis factor thrombospondin-1, vascular endothelial growth factor, and evidence of basal lamina reduplication (76). Angiogenesis, tumor necrosis (TN), epidermal growth factor (EGFR) expression, cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMPs) have all been linked to a worse outcome in some types of MM solid tumors (79).

1.4. Malignant effusions in serosal cavities

1.4.1. The clinical relevance of malignant effusions

Malignant effusions are caused by the escape of fluid from the blood or lymph vessels into tissues or cavities. It is a phenomenon that occurs frequently in patients with cancer, and it is not restricted to any specific form of cancer. Malignant effusions can occur in all types of cancer that has the potential to metastasize to any of the body's serous cavities (80). The serosal cavities include peritoneal, pleural and pericardial cavities, and the presence of cancer cells in effusions at these sites are evidence of advanced-stage disease with metastatic spread. Regardless of tumor site of origin, metastatic spread to the serosal cavities signifies disease progression and is linked to a worse prognosis and poor survival (81-82). The most frequent site of origin for tumor cells in effusions are primary carcinomas of the ovary, breast and lung, but the cells can also originate from MM which is the native tumor for the serosal cavities (83). The underlying mechanisms for the generation of effusion fluid within the serosal cavities are thought to be a combination of lymphatic obstruction by metastatic cancer cells, increased production of fluid by cells lining these cavities, and increased vascular permeability (82, 84-85). In the case of malignant effusions within the peritoneal cavity (ascites) the patient often presents with abdominal distension, weight gain, indigestion, dyspnea, orthopnea, and tachypnea. Nausea and vomiting can also occur as a result of intestinal obstruction due to large ascites (80). As malignant acsites cannot be surgically removed like its solid tumor counterparts, the standard treatment relies on diuretics and therapeutic paracentesis. In addition there is use of treatment modalities such as systemic and

intraperitoneal immunotherapy, permanent drains and peitoneovenous shunts (86).

Routine cytomorphology is commonly used for cytological diagnosis of serous effusions (87). In cases where there is a large number of cancer cells present in the serous effusion, the detection and diagnosis is a straightforward procedure. However if there are few atypical cells in addition to large number of reactive mesothelial cells (RM) and macrophages, obtaining an accurate diagnosis can be troublesome (81, 82). In addition, mesothelial cells react with to a wide variety of stimuli and injuries that break their continuity by proliferation and cellular changes, including marked nuclear and cytoplasmic alterations that can mimic the morphology of malignant cells (88). As the mixture of epithelial and mesodermal cells in both MM and OC have common embryonic origin, it is difficult to obtain an accurate diagnosis based on the effusion sample. Taken together with the fact that RM can be mistaken for MM, the most difficult differential diagnosis in effusion specimens is between metastatic OC and MM, and RM (82, 89).

Electron microscopy used to be the gold standard for effusion diagnosis. The method provides a high yield of valuable information, but there are some clear disadvantages such as the need for highly specialized personnel, considerable cost, and time-consuming procedures, which limit the use of the method to highly-specialized centers. As a result, other ancillary methods have been

employed, out of which IHC has emerged as the most frequently used method (89). Additional ancillary methods that have been used to increase the diagnostic accuracy of serous effusions are flow cytometry (FC), polymerase chain reaction (PCR), immunofluorescence, telomerase activity, fluorescent in situ hybridization and DNA ploidy analysis (81, 87, 89). More recent advances have introduced the use of molecular methods such as quantitative real-time PCR (qRT-PCR), comparative genomic hybridization (CGH), microarray and proteomics (83, 90-91).

1.4.2. Molecular alterations in effusions

Unlike their solid tumor counterparts, cancer cells in effusion are in a unique hypoxic microenvironment where there is limited access to nutrients and no presence of stromal myofibroblasts and endothelial cells that participate in paracrine tumor-promoting pathways. Based on these evident physical differences between cancer cells in solid tumors and in effusions, there are strong indications of differential expression of genes between these two counterparts. Studies have revealed that this is the case for several genes such as proteases (MMP-2, MMP-9, TIMP-2), cadherins (E-cadherin), growth factor receptors (TrkA, $p75^{NGFR}$), integrins (β1 integrin subunit), angiogenic molecules (VEGF, bFGF, IL-8, heparanase) and anti-apoptotic molecules (XIAP, Survivin) (reviewed in ref 82).

With focus in OC, a wide range of molecules have been investigated in relation to their differential expression in effusions compared with primary tumors. The results show that claudin-1, claudin-3, claudin-7, E-cadherin, catenins, Sip1, β1 integrin, MMP-2, Kallikrein 4 and $p75^{NGFR}$ are upregulated in OC effusions compared to solid tumors, whilst Slug, Snail, VEGF, IL-8, TIMP-2, GEP and TrkA are downregulated (92-94).

In relation to MM, studies have shown that MM effusions have upregulated levels of N-cadherin, P-cadherin and E-cadherin, XIAP and HLA-G compared to solid tumors. Whilst the opposite is shown to be the case for heparanase, bFGF, Survivin and Ki-67 that all have significantly lower expression levels in effusions compared to solid MM (83).

1.5. RAS superfamily of small GTPases

1.5.1. RAB GTPases

RAB GTPases are small GTP-binding proteins that form the largest family within the RAS superfamily of small GTPases (95). RAB GTPases are present in all eukaryotes, and over 60 human RAB genes have been identified (96-97). The various RAB GTPases are localized to the cytosolic face of specific intracellular membranes, where they serve as coordinators of vesicle traffic. The RAB proteins take part in control of membrane identity and regulate the functions of vesicle budding, uncoating, motility and fusion (96, 98). As regulatory proteins,

their mode of action is to alternate between two conformational states; the active GTP-bound form and the inactive GDP-bound form (95, 99).

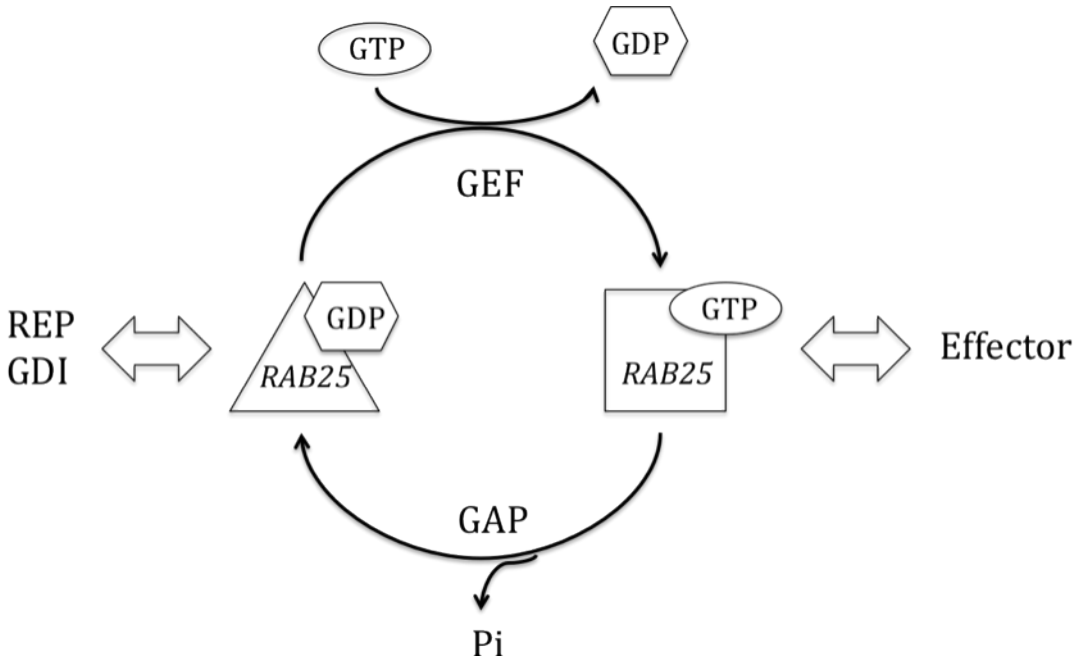


Figure 1: The Rab GTPase cycle.

Conversion of the RAB GTPase in its inactive GDP-bound form to its active GTP-bound form is caused by nucleotide exchange. The inactive GDP-bound form interacts with Rab escort protein (REP) and GDP-dissociation inhibitor (GDI). The molecular switch altering the inactive GDP-bound form into its active GTP-bound form is catalyzed by a GDP/GTP exchange factor (GEF). In their active form RAB GTPases are responsible for recruiting specific sets of effector proteins onto the specific membrane on which the RAB is located. It is through the action of these effector proteins that the RAB GTPases are capable of exerting their action as coordinators of vesicle traffic (95-96). The GTPase-activating protein

(GAP) is responsible for the GTP hydrolysis that releases inorganic phosphate (Pi), and causes cycling of RAB GTPases from their active GTP-bound form back to their inactive GDP-bound state (95, 98).

1.5.2. The role of RAB25 in cancer

Several RAB GTPases have been examined for being potential drivers in carcinogenesis, and RAB25 is one of them (100). In contrast to most of the RAB proteins that have a ubiquitous distribution, RAB25 is found to have an epithelial distribution (101). Together with its paralogues, RAB11a and RAB11b, RAB25 are located in polarized epithelial cells where exerts its function of regulating polarized endocytic recycling (102). Studies have shown that RAB25, located at chromosome1q22, is amplified at the DNA level and over-expressed at the RNA level in ovarian and breast cancer. In both malignancies these changes are associated with poor patient outcome. The underlying mechanisms of the poor prognosis are related to RAB25s intrinsic properties that promote anchorage-independent growth, decreased apoptosis and increased proliferation and aggressiveness *in vivo* (103). Increased RAB25 levels are also observed in other forms of cancer such as prostate cancer and transitional cell carcinoma of the bladder, providing further indication of RAB25s involvement in carcinogenesis (104).

1.6. Preferentially expressed antigen of melanoma (PRAME)

The preferentially expressed antigen of melanoma (PRAME) gene encodes an antigen presented to specific autologous cytotoxic T-lymphocytes (105). PRAME is a 509 amino acid protein originally identified as an antigen recognized by cytotoxic T lymphocytes from a melanoma patient (106-107). Most normal tissues do not express PRAME, the exception are testis and to an even lesser extent endometrium, ovary and adrenals. In addition there has been miniscule detection of PRAME expression in some samples originating from kidney, brain and skin (105).

1.6.1. The role of PRAME in cancer

There was no known function of PRAME until the discovery of PRAME acting as repressor of retinoic acid (RA) signaling (RAR). RA signaling is essential in development, cell fate determination and tissue homeostasis. By binding to its receptor and subsequent activation, RA initiates transcription of genes involved in differentiation and cell cycle arrest. The repressing action of PRAME on RA is therefore beneficial for tumor development (108-109).

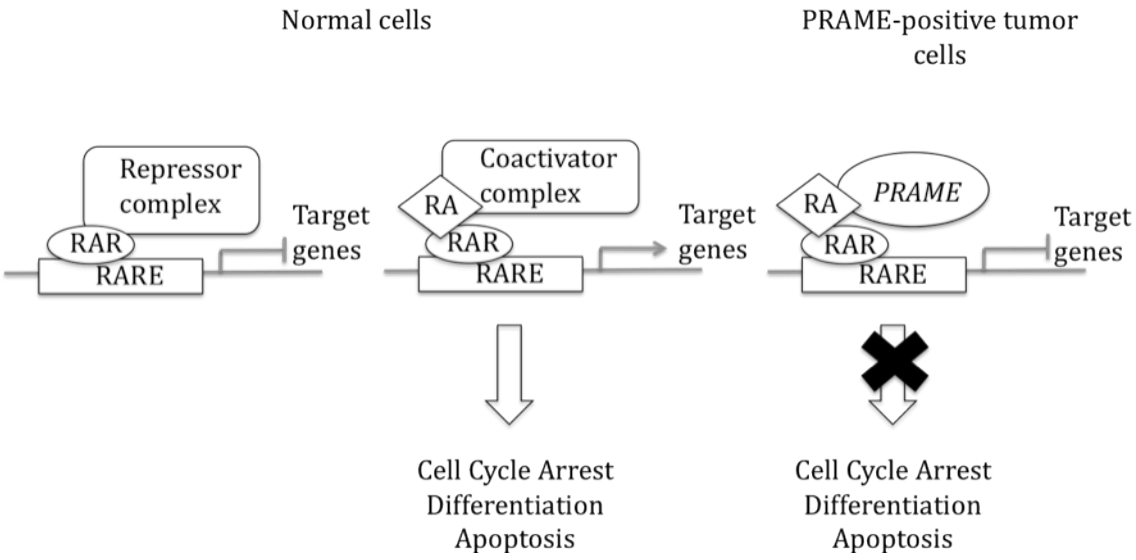


Figure 2: Model of PRAME function in cancer.

PRAME is expressed in various malignancies such as melanoma, non-small cell lung carcinomas, breast carcinomas, renal cell carcinomas, head and neck squamous cell carcinomas, renal carcinomas, Hodgkin's lymphomas, sarcomas, neuroblastomas, medulloblastomas and in the acute and chronic phases of both myeloid and lymphocytic leukemias (105, 109-111).

1.7. EHF (Ets homologous factor)

1.7.1. The ETS gene family

Environmental stimuli are able to induce cellular responses through the mechanism of signaling cascades. The interplay of ligand-activated cell surface receptors and the activation of downstream effector molecules lead to a

transduction of the extracellular signal from the cell surface to the nucleus. The ETS gene family of transcription factors is target for such signaling pathways, and the ETS genes are capable of directing cytoplasmic signals to control gene expression (112). The ETS gene family was discovered through the identification of the viral oncogene v-ets in the avian transforming retrovirus E26 (113-116). The gene family is one of the largest families of transcriptional regulators, and approximately 30 human ETS family members have been identified. As transcription factors, the ETS gene products are capable of activating or repressing expression of genes that are involved in various biological processes such as control of cellular proliferation, differentiation, hematopoiesis, apoptosis, tissue remodeling, angiogenesis, transformation, invasion and metastasis (113, 115-118). All ETS genes have an evolutionary conserved ETS domain consisting of an 85 amino acid region making up a DNA binding domain. This DNA binding domain recognizes a core GGAA/T sequence known as the Ets Binding Site (EBS), (113-116). All ETS transcription factors bind to the EBS, and such EBS have been identified in the promoter and enhancer regions of viral and cellular genes (Sementchenko 2000). Several Ets proteins also contain the Pointed (PNT) domain that plays a key role in protein-protein interaction (116). ETS gene family members affect an estimated 200 target genes, and the number of genes regulated via EBS is constantly increasing (117). EHF (ETS homolgous factor) is a transcription factor belonging to a specific group within the ETS gene family. EHF, also known as ESE-3, is located at 11p12 and transcribes a nuclear protein of 300 amino acids in size (115, 119). EHF is epithelial specific and has distinct functions in epithelial cell differentiation and proliferation. The highest

level of EHF expression is found in glandular epithelium such as prostate, pancreas, salivary gland, and trachea (120).

1.7.2. ETS factors and their role in cancer

As a result of their involvement in proliferation, differentiation and angiogenesis, ETS gene products are able to contribute to tumor growth and consequently disease progression. The ETS transcriptor factor family member ETS-1 is a proto-oncogene that is produced by a variety of tumors, and dependent on tumor type its expression is either increased or exclusively found in invasive higher grade tumors. High levels of ETS-1 expression are shown to correlate with poorer prognosis in OC (118, 121-124). Similarly, it has been shown that expression levels of PEA3, another member of the ETS transcription factor family is associated with tumor progression and poor survival in OC (125).

1.8. Methodological background

1.8.1. Quantitative real-time PCR

The polymerase chain reaction (PCR) is one of the most powerful methods in the field of molecular biology. By the use of PCR one is able to amplify specific sequenced of DNA or complementary DNA (cDNA) in order to obtain many thousands to a million copies out of a single copy. Traditional PCR uses endpoint detection of presence or amount of the specific DNA sequence. In contrast quantitative real-time PCR (qRT-PCR) measures amount of PCR product at each

cycle (126). Thus the process of amplification is measured in real-time by the use of fluorescent techniques. The amplification curve generated for each sample in the qRT-PCR run is used to quantify the initial amount of template molecules present in the sample (127).

Despite variation in qRT-PCR protocols among laboratories and scientists, the underlying principle for the method is the same and its concept is relatively simple. First step is that of reverse transcription, a process that utilizes the enzyme reverse transcriptase to convert RNA into cDNA. The cDNA can subsequently be used as template for the qRT-PCR reaction, where it will be amplified and detected through the means of sequence specific primers and probe (128).

The major advantages of qRT-PCR compared to other nucleic acid quantification methods are its wide dynamic range with detection covering over eight orders of magnitude. Compared to conventional PCR, qRT-PCR also has a significantly higher reliability of the results due to the fact that the entire amplification profile is monitored (127). In addition no post-PCR steps are required which eliminates the risk of cross-contamination due to PCR products, a prime consideration for diagnostic samples in a medical setting (129).

The qRT-PCR technology is heavily dependant upon several critical factors in order to generate optimal results. The primary consideration is to have high

quality sample material as input, thus sample storage and sample preparation are of great importance. Further considerations are choice of reverse-transcription primers, and primers and probe for PCR assay, all of which will influence the assay performance if not sufficiently specific or efficient. In addition great emphasis should be focused on data handling and statistical analysis, as incorrect handling will generate highly misleading results (130).

1.8.2. Immunohistochemistry

Immunohistochemistry (IHC) is based on the principle of using an antibody directed against an antigen present in the cell or tissue of interest, together with a method to visualize this complex (131). The technique is commonly used in combination with cytology in order to obtain an accurate diagnosis. The IHC the technique provides stains based on antigen-antibody reactions that show substances present within the cell or tissue of interest, this feature proves to be of great value when differentiating between neoplasms that share similar cytological features like in the case of mesotheliomas versus adenocarcinomas. The technique is relatively fast and easy to perform, and it does not require expensive equipment. However there are pitfalls to be aware of when using the technique, such as false-negative and false-positive results. False-negative results can be caused by overheating which destroys the antigenic determinants, over-fixation with formalin which creates cross-linkages that can mask the antigentc determinants, poor handling of reagents and poor specimen preservation, or improper antibody dilution. False-positive results can be caused by accepting nonspecific or background staining as a positive result (132).

2. AIMS OF THE STUDY

Based on a cDNA microarray study conducted by Davidson in 2006 where 189 genes were found to have differential expression between DMPM and OC/PPC specimens, there was a desire to investigate the expression pattern of some of these genes in more detail (133). Thus the aim for this project was to elucidate the gene expression levels of the three genes; *EHF, PRAME* and *RAB25* in a larger sample material consisting of OC and primary peritoneal carcinoma (PPC) vs. MM in serous effusions, by the means of qRT-PCR.

Consequently, the aims for this project were:

1. To develop qRT-PCR assays for the genes *RAB25* and *PRAME*. This includes designing primers, probes, and external standard (oligonucleotides), and optimizing the conditions for the qRT-PCR reaction.

2. To measure gene expression levels of *RAB25, PRAME* and *EHF* in OC/PPC and MM effusions, in order to validate the previously-pubished array data in a large sample material.

3. To compare the levels of the above-mentioned genes in primary OC vs. OC effusions, with the aim of defining the potential role in tumor progression.

4. To analyze and the clinical role of *RAB25, PRAME* and *EHF* mRNA and RAB25 protein expression in OC/PPC.

3. MATERIAL AND METHODS

3.1. Clinical material

The specimens studied in this project consisted of fresh (non-fixed) malignant effusions from patients diagnosed with OC/PPC and MM, as well as solid specimens of primary OC submitted for routine diagnostic purposes to the Department of Pathology, Norwegian Radium Hospital, Oslo University Hospital, during 1998-2005.

The project included qualitative real-time PCR analysis of a total of 112 fresh-frozen effusions, out of which 98 were OC/PPC and 14 were MM. OC/PPC effusions (n=98; 74 peritoneal and 24 pleural) were obtained from 93 patients (5 patients with 2 effusions each) diagnosed with advanced-stage (International Federation of Gynecology and Obstetrics stage III-IV) OC (n=77), PPC (n=11), or the closely related serous carcinoma of the fallopian tube (n=5). The majority of OC/PPC specimens (82/98; 84%) were of serous type. The 14 MM effusions consisted of 10 pleural and 4 peritoneal specimens. All were from patients diagnosed with MM of the epithelioid or biphasic type in biopsy specimens. In addition 28 solid primary OC were examined, all of which were of the serous type.

For IHC a total of 245 effusions (209 OC/PPC and 36 MM) were analyzed. All OC/PPC specimens were of the serous type and the MM effusions were from patients diagnosed with MM of the epithelioid or biphasic type in biopsy

specimens. A series of 101 serous carcinomas (34 primary carcinomas, 67 solid metastases) were additionally immunostained. The majority of metastases were omental. Specimens were patient-matched tumors from 46 of the patients with OC effusions studied. This series was in a tissue microarray containing 2-mm cores from patients operated at the Norwegian Radium Hospital, Oslo University Hospital, during 1998-2004.

3.1.1. Preparation of effusion specimens

In order to obtain maximal cell viability and to eliminate any bias caused by handling, a standard protocol was applied to the specimens immediately after tapping. The protocol includes a centrifugation of the cells at 2000 rpm for 10 minutes, followed by the preparation of two Diff-Quik®-stained and two PAP-stained smears. An experienced cytopathologist (Prof. Ben Davidson) approved the specimens that could be included in the study, and the criteria were that each specimen had to display a clear tumor cell population constituting at least 50% of the total cell population and be free from any degraded cells. Effusion pellets were divided in order to generate both formalin-fixed paraffin-embedded cell blocks and fresh-frozen material. Fresh-freezing was carried out at -70°C in RPMI 1640 medium containing 50% fetal calf serum (FCS) and 20% dimethylsulfoxide (DMSO) at a ratio of 1:1. The formalin-fixed paraffin-embedded cell blocks were prepared using the Thrombin clot method (134).

3.1.2. Preparation of tissue

Surgical specimens were snap-frozen and kept at -70°C with no medium. A frozen section was then carried out on the solid tumors to ensure a tumor cell content of at least 50% and to exclude the presence of necrosis.

3.1.3. Pathological diagnosis

Experienced cytopathologists and surgical pathologists undertook the pathological diagnosis on all the specimens used in the study. In cases which were newly diagnosed as cancer based on cytology or histology specimens, a second senior pathologist confirmed the diagnosis. Smears and H&E-stained sections from all effusions and H&E-stained slides from all solid tumors were reviewed by Prof. Ben Davison.

3.1.4. Clinical data

All relevant clinical data including patient age, FIGO stage, residual disease volume, dates of chemotherapy administration and clinical response to treatment, as well as progression-free survival (PFS) and overall survival (OS) data for the OC/PPC specimens were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital, Oslo University Hospital.

3.1.5. Ethics

This project was approved by the Regional Committee for Medical Research Ethics Norway (S-04300). Norwegian laws and rules, including Biobank Law and Data Inspectorate Regulations, were strictly followed.

3.2. Isolation of RNA and cDNA synthesis

The fresh-frozen effusion specimens were dissolved in 15 ml cold phosphate buffered saline (PBS) and centrifuged at 1200 rpm for 5 minutes at 4°C. After centrifugation the pellet was resuspended in 2 ml PBS and before an additional centrifugation with the same parameters. Following the second centrifugation the supernatant was discarded, and pellet was used as input material for the Qiagen protocol "Isolation of total RNA from cells" using RNeasy Mini Kit®. The fresh-frozen tissue samples were manually cut into the appropriate size of 27mm^3 (3 mm^3 cube) in order to comply with the instructions stated in the Qiagen "RNeasy Fibrous Tissue" protocol of using 25-35 mg tissue input. The procedure was carried out on dry ice to ensure proper cooling of the tissue in order to prevent RNA degradation. Tissue specimens were placed in lysis buffer and an electrical homogenizer (TissueRuptor®, Qiagen) was used to homogenize the tissue. Isolation of total RNA from both the fresh-frozen effusion and the frozen tissue specimens was carried out by the use of an automated sample preparation instrument (QiaCube®, Qiagen).

The RNA concentrations of all samples, along with the 260 nm/280 nm and 260 nm/230 nm ratio were measured by a BioPhotometer 6131 instrument (Eppendorf). All nucleotides (RNA, ssDNA and dsDNA) absorb at 260 nm, whereas protein, phenol or other contaminants absorb at 280 nm. A 260 nm/280 nm ratio of ~ 2.0 is generally accepted for pure RNA. The 260 nm/ 230 nm ratio is used as a secondary measurement of nucleic acid purity. The wavelength of 230 nm is near the absorbance maximum of peptide bonds, and it also indicates buffer contamination since EDTA and other buffer salts absorb at this wavelength. The 260 nm/230 nm for pure nucleic acids are often higher than the respective 260 nm/280 nm, with expected values commonly being in the range of 2.0-2.2 (135).

RNA was transcribed into cDNA by the use of Superscript III Reverse Transcriptase (Invitrogen, Carlsbad CA). The various RNA concentrations of the samples were normalized in order to obtain cDNA with a concentration of 20ng/ul in a total volume of 150 ul (3000 ng in 150 ul). The samples were normalized using DEPC-treated water to obtain a total volume of 20 ul. The 20 ul of normalized sample was incubated at 70°C for 10 min, before it was mixed with 20 ul of cDNA synthesis mastermix containing 5x First-Strand Buffer, 0.1M DTT, 10mM dNTP mix, random primers (50 ng/ul), RNaseOUT 40U/ul, and Superscript III 200U/ul with the following endpoint concentrations per reaction of 1x, 10 mM, 1 mM, 25 uM, 20 U and 100 U respectively. The cDNA synthesis program was as follows:

18°C for 10 min, 50°C for 45 min, 99°C for 3 min and 4°C as a hold temperature. Upon completion of cDNA synthesis the cDNA was diluted with 110 ul of MilliCure water in order to get the total volume of 150 ul.

3.3. Quantification of gene expression levels with qRT- PCR

3.3.1. Designing primers and probes for qRT- PCR

Three gene-specific primer pairs with accompanying probe was designed for each of the genes; *EHF, PRAME* and *RAB25*. When designing primers and probes several considerations were taken. An important feature was the location of the primers or probe within the gene sequence. The location should be so that either one of the primers or the probe were positioned over an exon-exon junction in order to rule out any binding to genomic DNA and thus ensure that the primer or probe would only bind to the cDNA synthesized from the spliced mRNAs. Ensembl was used to view positions and sequences for the exons and introns of the various genes (http://www.ensembl.org/index.html). Another consideration was that the primers should have a melting temperature (Tm) just below 70°C, and the probes a Tm just below 80°C. The Tm calculator from Finnzymes was used to design primers and probes with the desired Tm (https://www.finnzymes.fi/tm_determination.html). A further consideration was the length of the primers and probes. In order to create an assay with a standard curve generated by the use of synthesized oligonucleotides, the total bp length of the sequence; forward primer - intervening bp's – probe - intervening bp's - reverse primer, could not exceed a total length of 100 bp as this was the maximum length of the synthesized oligonucleotides. After these parameters

were met the primer and probe sequences were checked for primer secondary structures; hairpins, dimers and crossdimers. This was done by the use of Premiers Biosoft International's primer analysis software NetPrimer (http://www.premierbiosoft.com/netprimer/index.html). Primers and probes were also checked for cross homology with any other genes in order to ensure that the primers would only amplify the specific gene of interest. The nucleotide BLAST on the NCBI webpage was used for this purpose (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The NCBI database was referred to in order to avoid single nucleotide polymorphisms (SNPs). The three gene-specific primer pairs and accompanying probe was synthesized by Invitrogen, Carlsbad CA.

3.3.2. Validating primers and probes

The validation process of the candidate primers and probes for all three genes involved multiple steps. The first step was to test the newly designed primers for *RAB25* and *PRAME* in a polymerase chain reaction (PCR) and apply the PCR product on an agarose gel to observe if there had been successful amplification. Primer specificity was validated by running in parallel genomic DNA and cDNA as template, and including control assays for genomic DNA (*KIT* assay) and cDNA (*GUS* assay). The reagents used for the PCR was Phusion HotStart enzyme (2U/ul), 5x HighFidelity Buffer, dNTP (10 mM), forward primer and reverse primer (20 uM), and dH2O at the concentrations of 0.4 U, 1x, 200 uM (50 uM each), 480 nM and 480 nM, respectively. Template amount used was 2.5 ul cDNA (20 ng RNA/ul). The following PCR program was used: 98°C for 2 min, 35 cycles

of 98°C for 20 sec, 60°C for 20 sec, 72°C for 20 sec, cycling followed by 72°C for 1 min and 4°C as a hold temperature. An agarose gel of 2% was made by dissolving 0.8 g Seakem agarose in 40 ml of TBE 0.5X, and adding 2ul SYBRsafe for DNA staining. The PCR products (10 ul) were mixed with loading buffer (2 ul) and together with a 1 kb ladder (4 ul) the specimens were loaded on to the gel and run at 90 V for 30 min.

Once the various candidate assay primers had been confirmed functional, the primers were tested for efficiency on real-time PCR by the use SYBR Green chemistry. Synthetic oligonucleotides were used as standards for the quantitative real time PCR. These synthetic oligonucleotides were designed to span the region of interest, consisted of a 100 bp, and were ordered from Invitrogen. BioMek 3300 automated pipetting robot (Beckman Coulter ™) was used to generate the dilution series of the standards, and the pipetting robot was also used to set up all assays that were analyzed on the LightCycler480 real time PCR instrument (Roche Diagnostics, Basel, Switzerland). The reagents used for the PCR were 2x SYBR Green PCR Master Mix, forward primer and reverse primer (20 uM), and dH20 at the following concentrations per reaction; 1x and 150 nM. The SYBR Green analysis allowed for discrimination between the candidate assay primers, and a decision was made on which of the primers that had the best efficiency on qRT-PCR. The corresponding TaqMan probe for the chosen assay was ordered from Invitrogen (Carlsbad, CA), and the next step was to perform a final test on the complete candidate assay for each of the three genes. See table 3 for primers, probe and oligonucleotides used for qRT-PCR.

Table 3: Primers, probe and oligonucleotides designed for qRT-PCR

Gene	Sequence (5'→3')	Amplicon length
RAB25		
Forward primer, exon 1	GGGGAATGGAACTGAGGAAGATTATAACT	
Reverse primer, exon 1	CGTGAATCGGGAGAGTAGATTGGTC	97 bp
Probe, exon 1-2	CAAGGTGGTGCTGATCGGCGAATCA	
Oligonucleotide forward, exon 1-2	ATGGGGAATGGAACTGAGGAAGATTATAACTTTGTCTTCAA GGTGGTGCTGATCGGCGAATCAGGTGTGGGGAAGACCAATC TACTCTCCCGATTCACGC	
Oligonucleotide reverse, exon 1-2	GCGTGAATCGGGAGAGTAGATTGGTCTTCCCCACACCTGAT TCGCCGATCAGCACCACCTTGAAGACAAAGTTATAATCTTC CTCAGTTCCATTCCCCAT	
PRAME		
Forward primer, exon 4-5	CTGGATCAGTTGCTCAGGCA	
Reverse primer, exon 5	CATCACATCCCCTTCCGAAAG	84 bp
Probe, exon 5	TGATGAACCCCTTGGAAACCCTCTCAATAACTAACTGCC	
Oligonucleotide forward, exon 4-5	AGAGGCCGCCTGGATCAGTTGCTCAGGCACGTGATGAACC CCTTGGAAACCCTCTCAATAACTAACTGCCGGCTTTCGGAA GGGGATGTGATGCATCTGT	
Oligonucleotide reverse, exon 4-5	ACAGATGCATCACATCCCCTTCCGAAAGCCGGCAGTTAGTT ATTGAGAGGGTTTCCAAGGGGTTCATCACGTGCCTGAGCAA CTGATCCAGGCGGCCTCT	
EHF		
Forward primer, exon 1-2	ACCCAGAATCTTTAGGTAAATGAGATC	
Reverse primer, exon 1-2	CTGGTGAAGGAGGTTGTTGC	88 bp
Probe, exon 1-2	ATTCTGGAAGGAGGTGGTGTAATGAATCTCAACC	
Oligonucleotide forward, exon 1-2	GATTTCCCACCCAGAATCTTTAGGTAAATGAGATCATGATT CTGGAAGGAGGTGGTGTAATGAATCTCAACCCCGGCAACA ACCTCCTTCACCAGCCGCC	
Oligonucleotide reverse, exon 1-2	GGCGGCTGGTGAAGGAGGTTGTTGCCGGGGTTGAGATTCA TTACACCACCTCCTTCCAGAATCATGATCTCATTTACCTAA AGATTCTGGGTGGGAAATC	

3.3.3. Quantitative real-time PCR

In order to quantify the expression of the genes of interest, qRT-PCR was performed using TaqMan probes labeled with the fluorescent label FAM. The LighCycler480 real-time PCR instrument (Roche Diagnostics, Basel, Switzerland)

was used, together with Platinum qPCR Supermix-UDG with ROX, $MgCl_2$ (50 mM), primers and probe for the specific assays (Invitrogen, Carlsbad, CA). The qRT-PCR reactions were set up in a 384-plate by the use of the BioMek 3300 automated pipetting robot (Beckman Coulter ™). Each qRT-PCR reaction contained 6.25 ul Platinum qPCR Supermix-UDG with ROX, 0.75 ul $MgCl_2$ (50 mM), 0.1875 ul of each primer (20 uM), 0.25 ul probe (5uM), 2,375 ul dH2O and 2.5 ul template (cDNA), making up a total volume of 12.5 ul. In the case of the standards the template cDNA was substituted with synthetic oligonucleotides diluted to the following concentrations; 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies per 5 ul. Each assay also include non-template controls where neither cDNA nor oligonucleotides where added to the mastermix. The following qRT-PCR program of 50 cycles was used: 50°C for 2 min, 95°C for 2 min followed by 95°C for 15 sec and 60°C for 1 min. A previous study of 12 reference genes (TaqMan low density array human endogenous control panel; Applied Biosystems) has been carried out in order to identify the most uniformly expressed transcript in effusion specimens. The results pointed out β-glucuronidase (*GUS*) as the optimal reference gene. Based on these results the expression levels of *RAB25, PRAME* and *EHF* were normalized against the housekeeping gene *GUS.* The *GUS* primers and probe have been published previously (136). Standards applied for the *GUS* reference assay was purchased from Ipsogen standards and cover the range of 10^2 to 10^5 copies (Ipsogen Cancer Profiler, Marseille, France).

3.4. Immunohistochemistry

Immunohistocehmistry (IHC) was performed to analyze RAB25 protein expression in effusions/solid tumors. In brief, paraffin-embedded sections were subjected to deparaffinization and epitope retrieval by the use of the Dako PT-Link (Dako, Glostrup, Denmark.) with a High-pH (50x TrisEDTA buffer pH 9) target retrieval solution (Dako). The sections were then incubated with 0.03% hydrogen peroxide to inhibit endogenous peroxidase. Afterwards the sections were incubated for 30 min with a commercially obtained mouse polyclonal anti-RAB25 antibody (Novus Biologicals, Littleton, CO, USA) diluted 1:500. Visulaization was achieved using EnVision™ FLEX+ peroxidase system (Dako). Positive control for RAB25 protein expression consisted of OC sections previously shown to express RAB25 protein. Negative controls consisted of sections that underwent similar staining procedure with normal IgG of the corresponding isotype as the primary antibody. Staining extent was was scored by a experienced cytopathologist (Prof. Ben Davison) using a scale of 0 to 4, corresponding to percentage of immunoreactive tumor cells of 0%, 1% to 5%, 6% to 25%, 26% to 75% and 76% to 100%, respectively. 100 out of the 346 samples were scored together with the author of this thesis, and interobserver concordance was excellent, with 90% total agreement on score. In cases with discordant scores, the difference was one of scoring level and consensus was easily reached upon discussion.

3.5. Statistical analysis

Statistical analysis was carried out using the SPSS-PC package (Version 15.0 and 16.0, Chicago, IL) by Prof. Ben Davison. Probability of <0.05 was considered significant. The Mann-Whitney U test was used to analyze the differences in gene expression between OC/PPC and MM, as well as to compare expression in solid primary OC and OC/PPC effusions. The Mann-Whitney U test was also applied to analyses investigating the association between gene expression and cliniopathologic parameters (effusion site, age, histological grade, FIGO stage, residual disease volume, previous chemotherapy, response to chemotherapy at diagnosis and at first disease recurrence) in OC/PPC. For these analyses, clinopahtologic parameters were grouped as follows: age, ≤60 versus >60 years; histological grade, 1-2 versus 3; FIGO stage, III versus IV; residual disease volume, ≤1 cm versus >1 cm; previous chemotherapy, yes versus no; response to chemotherapy for primary disease and for disease recurrence, complete versus partial response/stable disease/progression/allergic or adverse reaction. Survival data were available for all OC/PPC patients. Wilcoxon Signed Ranks Analysis was used to examine all paired data; primary OC and solid metastases, and effusions and solid primary or metastatic lesions. Univariate survival analyses of progression-free survival (PFS) and overall survival (OS) were executed using the Kaplan-Meier method and groups compared with the log-rank test. For this analysis expression levels were grouped as low or high based on median values in the case of qRT-PCR (mRNA) data, and focal (≤25% of cells) or diffuse (>25% of cells) in terms of IHC (protein) data. For patients with more than 1 effusion, expression in the first specimen was analyzed. Multivariate

analyses for OS and PFS were performed using the Cox proportional hazard model (Method=Enter).

4. Results

4.1. Assay design for *RAB25*

4.1.1. Testing of *RAB25* primer combinations with conventional PCR and agarose gel electrophoresis

The PCR products generated from the three primer pair combinations for *RAB25*; 2054+2055, 2057+2058 and 2076+2058 were run on a 2% agarose gel to test the different primer combinations ability to generate a PCR product.

The *RAB25* primer combination 2054+2055 generated a single band corresponding to 100 bp. The same observation was made for *RAB25* primer combination 2057+2058. However**,** this primer combination generated an additional band corresponding to 850-900 bp along with several bands in the range of 400-800 bp (Figure 3). The *RAB25* primer combination 2076+2058 generated one band corresponding to 100 bp, one band corresponding to 800 bp and several additional bands in the range of 600-800 bp (Figure 4). Both gels showed a strong band at approximately 200 bp indicating amplification of genomic DNA in the *KIT* gene assay, along with a band of 100 bp indicating amplification of cDNA in the *GUS* gene assay (Figure 3 and 4).

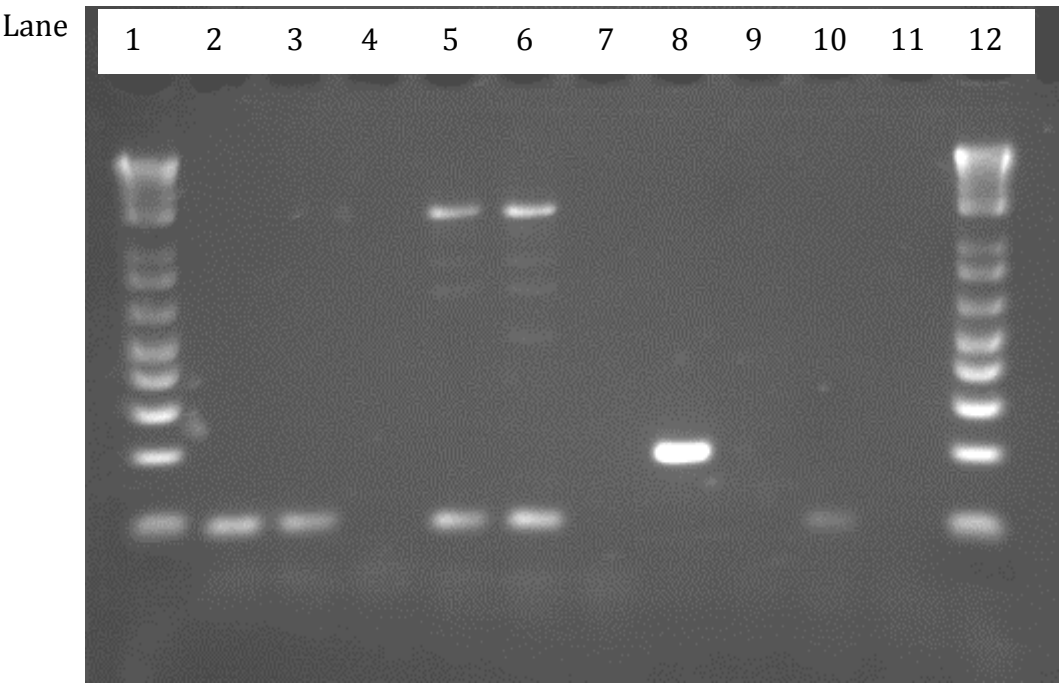


Figure 3: Agarose gel electrophoresis of PCR products. Samples were electrophoresed in a 2% agarose gel for 30 min at 90 V. Lane 1: 100 bp ladder (Invitrogen, Carlsbad CA). Lane 2 and 3: PCR product of *RAB25* primers 2054+2055. Lane 4: Negative control of *RAB25* primers 2054+2055. Lane 5 and 6: PCR product of *RAB25* primers 2057+2058. Lane 7: Negative control of *RAB25* primers 2057+2058. Lane 8: PCR product of primers amplifying *KIT* gene to check for presence of genomic DNA. Lane 9: Negative control for *KIT*. Lane 10: PCR product of primers amplifying expressed *GUS* gene. Lane 11: Negative control for *GUS*. Lane 12: 100 bp ladder (Invitrogen, Carlsbad CA).

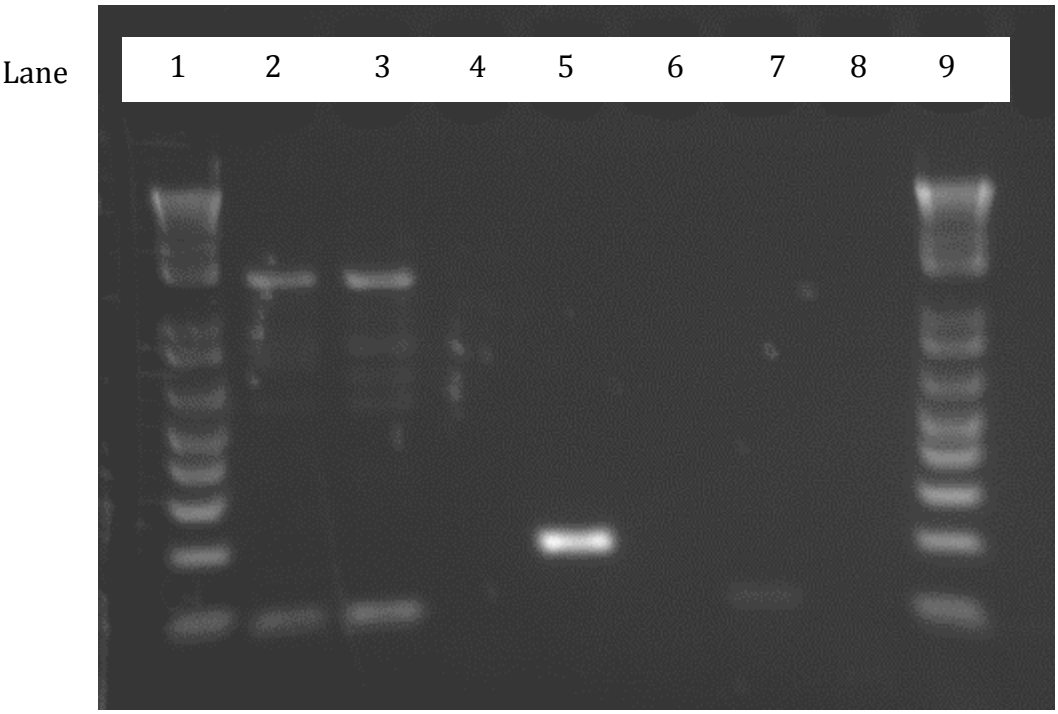


Figure 4: Agarose gel electrophoresis of PCR products. Samples were electrophoresed in a 2% agarose gel for 30 min at 90 V. Lane 1: 100 bp ladder (Invitrogen, Carlsbad CA). Lane 2 and 3: PCR product of *RAB25* primers 2076+2058. Lane 4: Negative control of *RAB25* primers 2054+2058. Lane 5: PCR product of primers amplifying *KIT* gene to check for presence of genomic DNA. Lane 6: Negative control for *KIT*. Lane 7: PCR product of primers amplifying expressed *GUS* gene. Lane 8: Negative control for *GUS*. Lane 9: 100 bp ladder (Invitrogen, Carlsbad CA).

4.1.2. Testing of *RAB25* primer combinations with SYBR Green

Both synthetic oligonucleotide combinations for *RAB25*; 2072+2073 and 2074+2075 were mixed and diluted to generate standards ranging from 10^6 to 10 copies per 5 ul. For the real-time PCR assays using either SYBR Green or

TaqMan probe, 2.5 ul synthetic oligonucleotides standards were used giving a copy number in the reactions ranging between 500,000 copies and 5 copies.

Primer combination 2054+2055 was tested together with the synthetic oligonucleotide standards generated from synthetic oligonucleotides 2072+2073, and primer combination 2057+5058 and 2076+2058 were tested together with the synthetic oligonucleotide standard generated from synthetic oligonucleotides 2074+2075. The results of the SYBR Green qRT-PCR assay with *RAB25* primer combination 2054+2055 on synthetic oligonucleotide standards 2072+2073 show a good differentiation from 500,000 to 50 copies. There is poor differentiation in the lowest range of the scale, i.e. 50 to 5 copies (Figure 5). The same is observed for the SYBR Green qRT-PCR assay with *RAB25* primer combination 2057+2058 on synthetic oligonucleotide standards 2074+2075 , where there is good differentiation from 500,000 to 50 copies but poor differentiation in the lowest range of the scale; 50 to 5 copies (Figure 6). The results of the SYBR Green qRT-PCR assay with *RAB25* primer combination 2076+2058 on synthetic oligonucleotide standards 2074+2075 show a clear generation of primer dimers and the result is no differentiation below 50,000 copies (Figure 7).

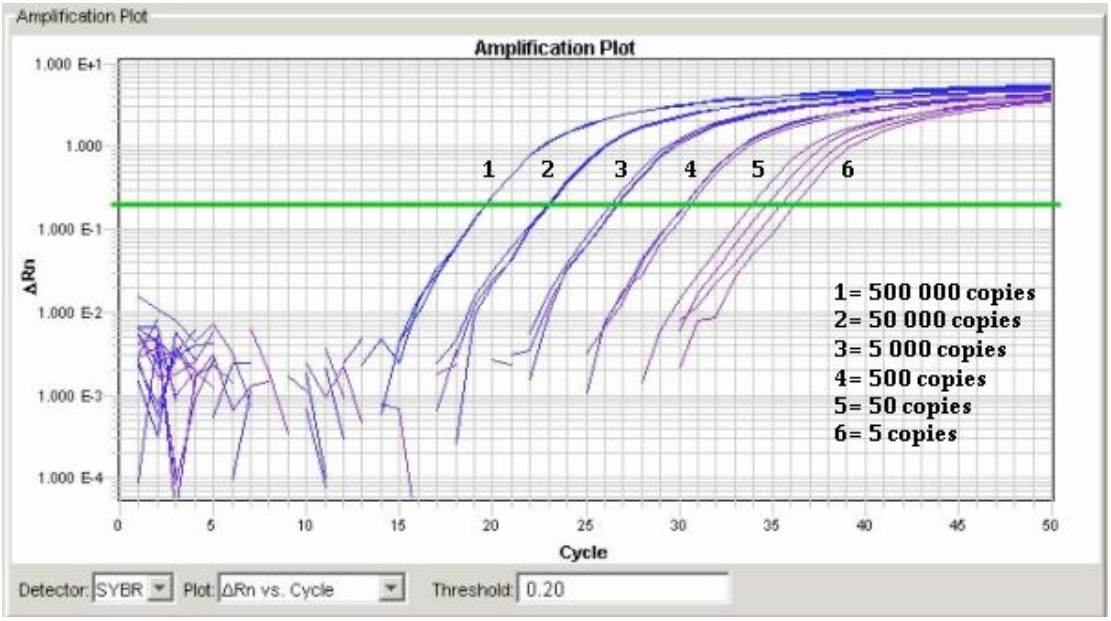


Figure 5: Amplification curves from SYBR Green qRT-PCR run with *RAB25* primers 2054+2055 on synthetic oligonucleotide standards 2072+2073.

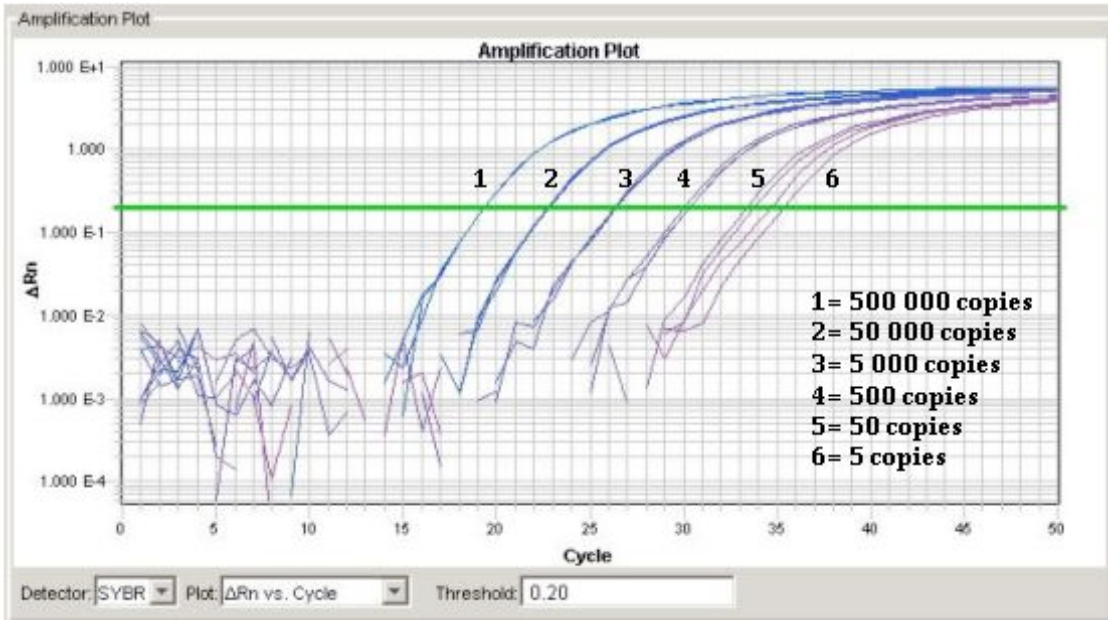


Figure 6: Amplification curves from SYBR Green qRT-PCR run with *RAB25* primers 2057+2058 on synthetic oligonucleotide standards 2074+2075.

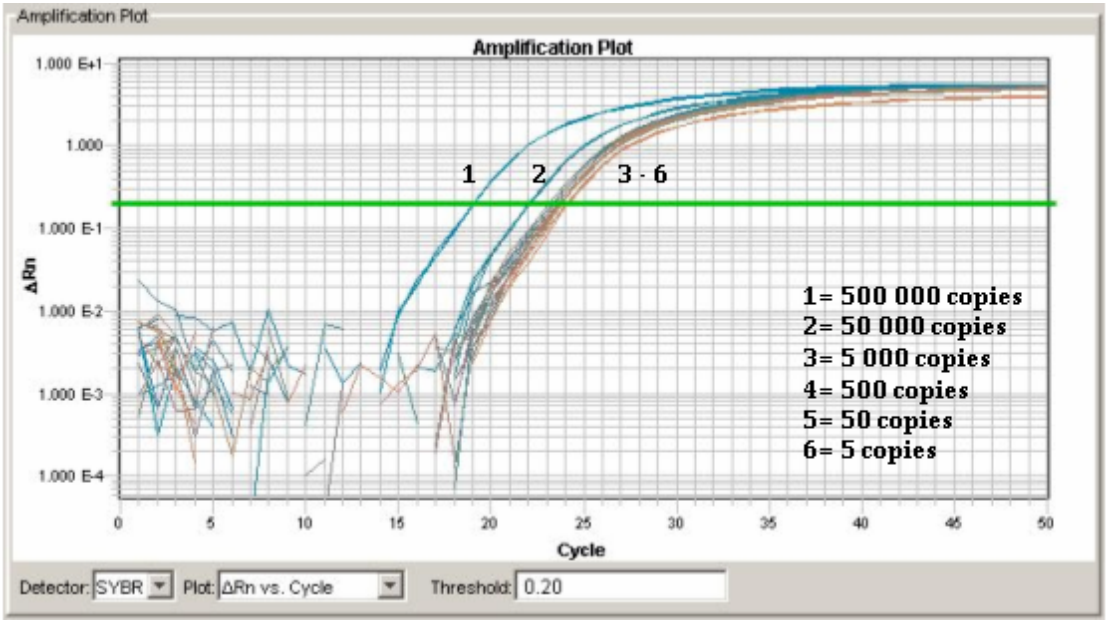


Figure 7: Amplification curves from SYBR Green qRT-PCR run with *RAB25* primers 2076+2058 on synthetic oligonucleotide standards 2074+2075.

4.1.3. Testing of RAB25 primer combination with TaqMan probe

The TaqMan probe corresponding to *RAB25* primer combination 2054+2055; 2056 was ordered and tested in a qRT-PCR run.

The results of *RAB25* qRT-PCR assay with primer combination 2054+2055 together with TaqMan probe 2056 on oligonucleotide standards 2072+2073 show good differentiation from 500,000 to 50 copies. However, there is very little differentiation between 50 to 5 copies (Figure 8).

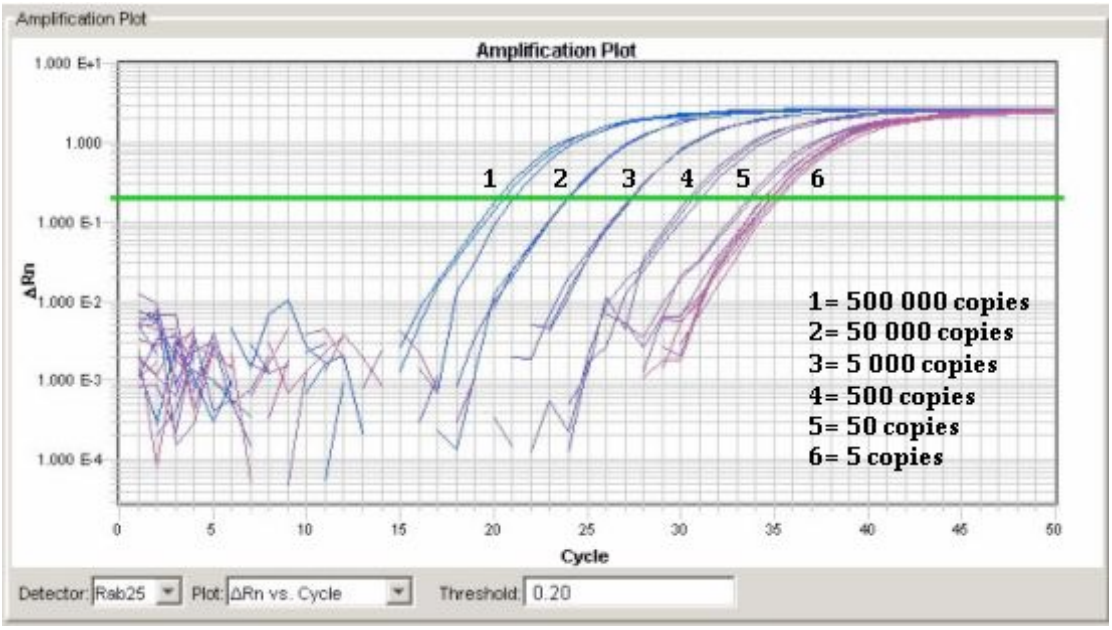


Figure 8: Amplification curves of *RAB25* on qRT-PCR using primers 2054+2055 together with TaqMan probe 2056 on synthetic oligonucleotide standards 2072+2073.

4.2. Assay design for *PRAME*

4.2.1. Testing of *PRAME* primer combinations with conventional PCR and agarose gel electrophoresis

As for *RAB25, t*he PCR products generated from the three primer pair combinations for *PRAME*; 2062+2064, 2065+2067 and 2068+2070 were run on a 2% agarose gel to test the different primer combinations ability to generate a PCR product.

Both *PRAME* primer combinations 2062+2064 and 2065+2067 generated one single band corresponding to 100 bp. In contrast the *PRAME* primer combination

2068+2070 generated an additional intense band corresponding to 800 bp. The gel showed a strong band at approximately 200 bp indicating amplification of genomic DNA in the *KIT* gene assay, along with a weak band of 100 bp indicating amplification of cDNA in the *GUS* gene assay (Figure 9).

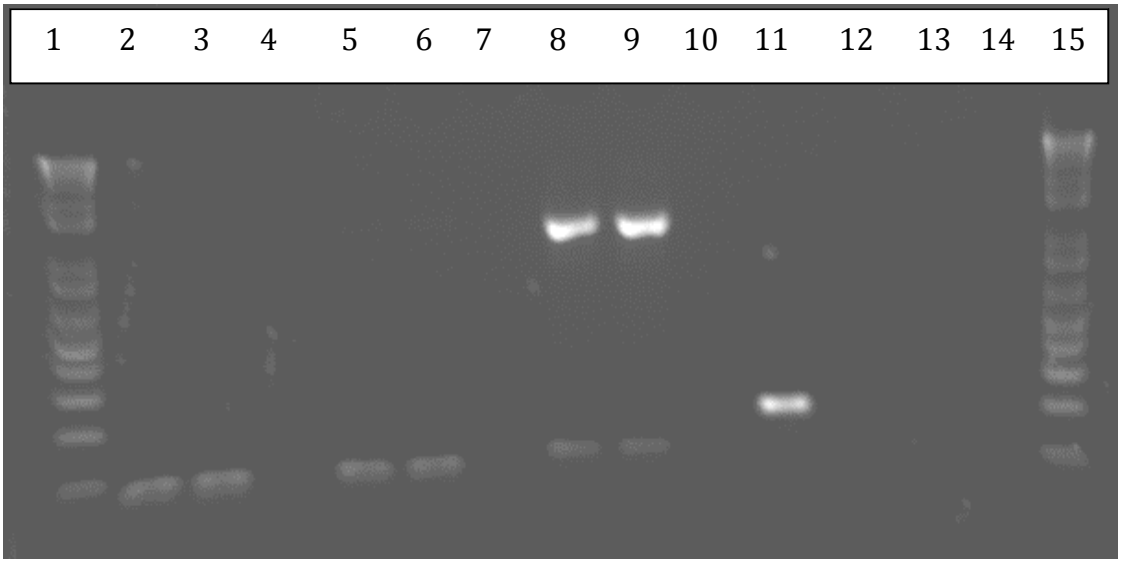


Figure 9: Agarose gel electrophoresis of PCR products. Samples were electrophoresed in a 2% agarose gel for 30 min at 90 V. Lane 1: 100 bp ladder (Invitrogen, Carlsbad CA). Lane 2 and 3: PCR product of *PRAME* primers 2062+2064. Lane 4: Negative control of *PRAME* primers 2062+2064. Lane 5 and 6: PCR product of *PRAME* primers 2065+2067. Lane 7: Negative control of *PRAME* primers 2065+2067. Lane 8 and 9: PCR product of *PRAME* primers 2068+2070. Lane 10: Negative control of *PRAME* primers 2068+2070. Lane 11: PCR product of primers amplifying *KIT* gene to check for presence of genomic DNA. Lane 12: Negative control for *KIT*. Lane 13: PCR product of primers amplifying expressed *GUS* gene. Lane 14: Negative control for *GUS*. Lane 15: 100 bp ladder (Invitrogen, Carlsbad CA).

4.2.2. Testing of *PRAME* primer combinations with SYBR Green

Both synthetic oligonucleotide combinations for *PRAME*; 2090+2091 and 2092+2093, were mixed and diluted to generate standards ranging from 500,000 copies to 5 copies.

Primer combination 2062+2064 was tested together with the synthetic oligonucleotide standards generated from synthetic oligonucleotides 2090+2091, whilst primer combination 2065+5067 was tested together with the synthetic oligonucleotide standards generated from synthetic oligonucleotides 2092+2093.

The results of the SYBR Green qRT-PCR assay with *PRAME* primer combination 2062+2064 on synthetic oligonucleotide standards 2090+2091 showed a good differentiation between 500,000 to 50 copies. There is poor differentiation in the lowest range of the scale, i.e. 50 to 5 copies (Figure 10). As for the SYBR Green qRT-PCR assay with *PRAME* primer combination 2065+2067 on synthetic oligonucleotide standards 2092+2093 show a good differentiation from 500,000 to 5 copies. However, there is a large spread in the triplicates for the 5 copies standard (Figure 11).

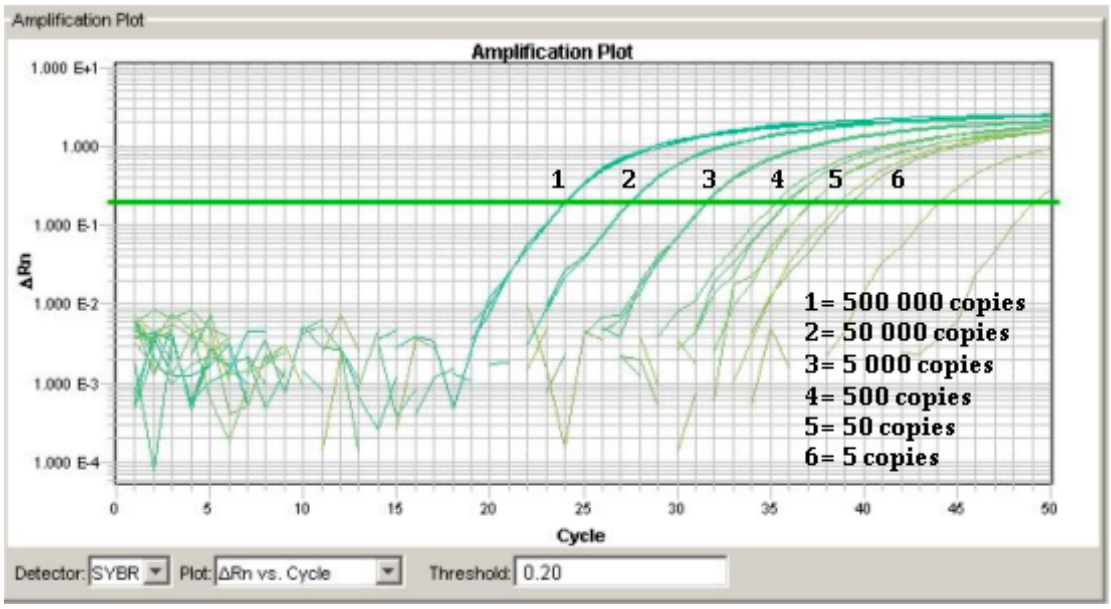


Figure 10: Amplification curves from SYBR Green qRT-PCR run with *PRAME* primers 2062+2064 on synthetic oligonucleotide standards 2090+2091.

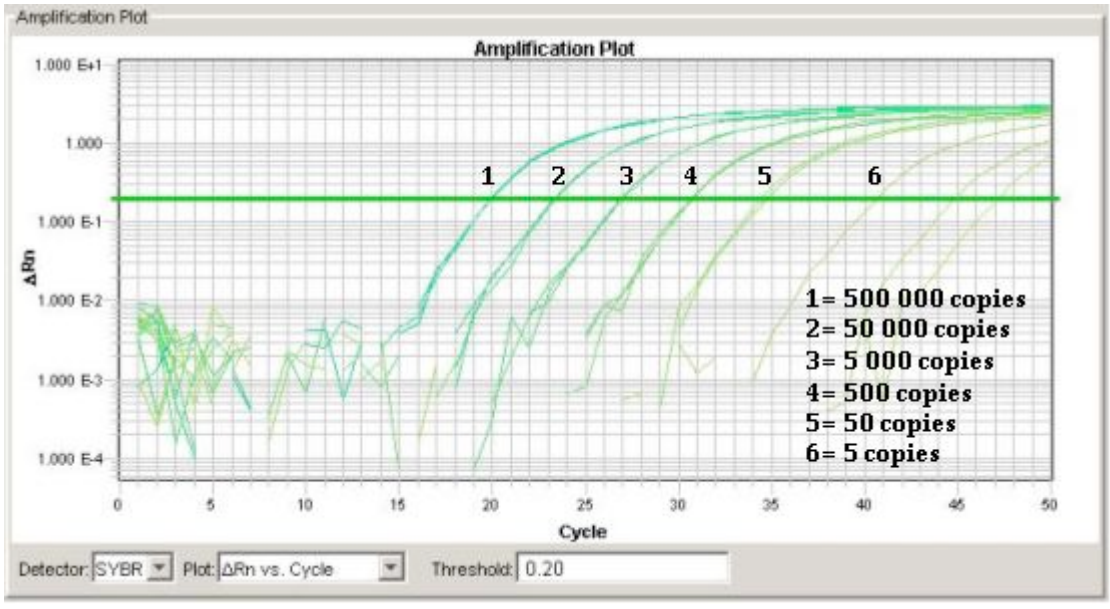


Figure 11: Amplification curves from SYBR Green qRT-PCR run with *PRAME* primers 2065+2067 on synthetic oligonucleotide standards 2092+2093.

4.2.3. Testing of *PRAME* primer combination with TaqMan probe

The TaqMan probe corresponding to *PRAME* primer combination 2065+2067; 2066 was ordered and tested in a qRT-PCR run.

The results of *PRAME* qRT-PCR assay with primer combination 2065+2067 together with TaqMan probe 2066 on synthetic oligonucleotide standards 2092+2093 showed good differentiation from 500,000 to 50 copies. However there was very little differentiation between 50 and 5 copies (Figure 12).

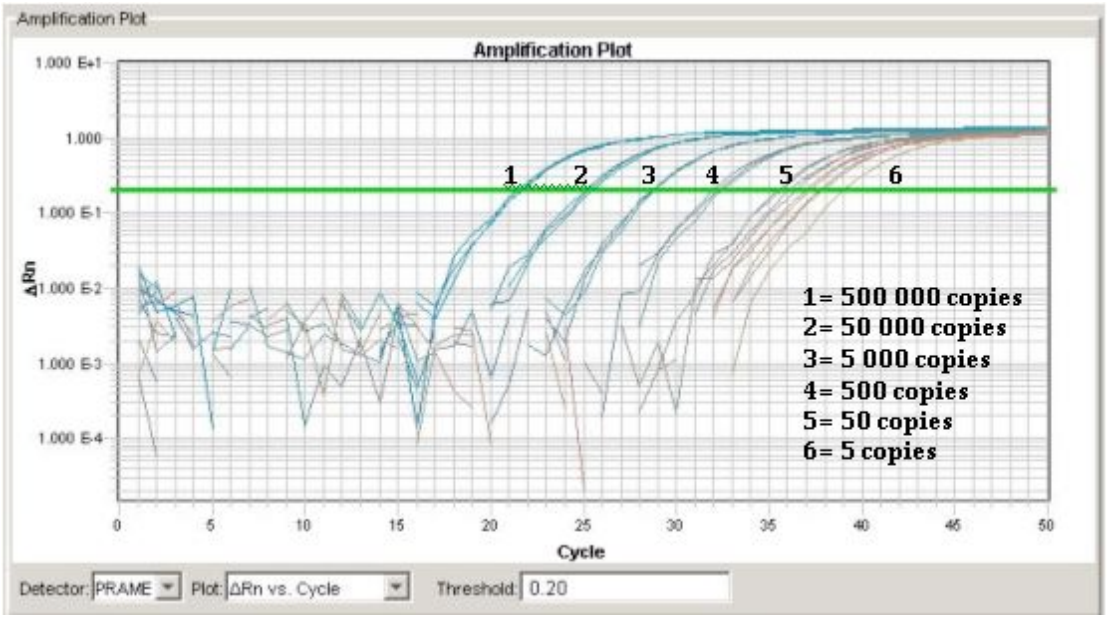


Figure 12: Amplification curves of *PRAME* on qRT-PCR using primers 2065+2067 together with TaqMan probe 2066 on synthetic oligonucleotide standards 2092+2093.

4.3. Gene expression levels of *RAB25, PRAME, EHF* and *GUS* in primary OC, OC/PPC effusions and MM effusions

The qRT-PCR assays showed that expression levels for *RAB25, PRAME* and *EHF* are highly variable within the primary OC, OC/PPC effusions and the MM effusions, whereas the reference gene *GUS* is uniformly expressed within the three different sample groups (Figure 13, 14 and 15).

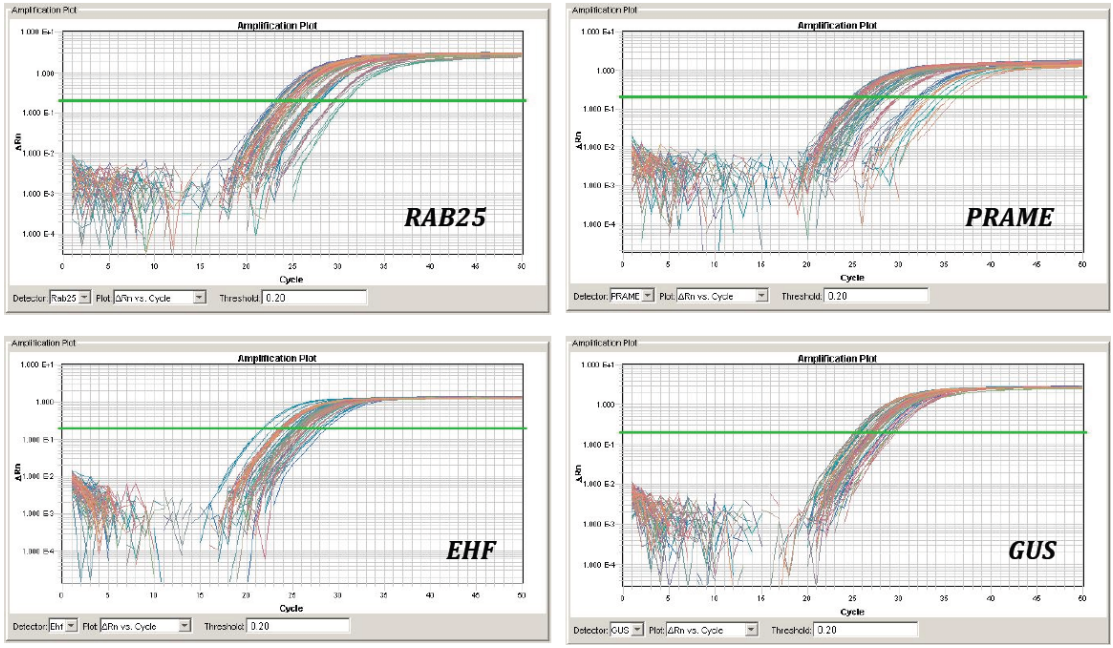


Figure 13: qRT-PCR plots for *RAB25, PRAME, EHF* and *GUS* mRNA expression in 28 primary OC, cases run in triplicate.

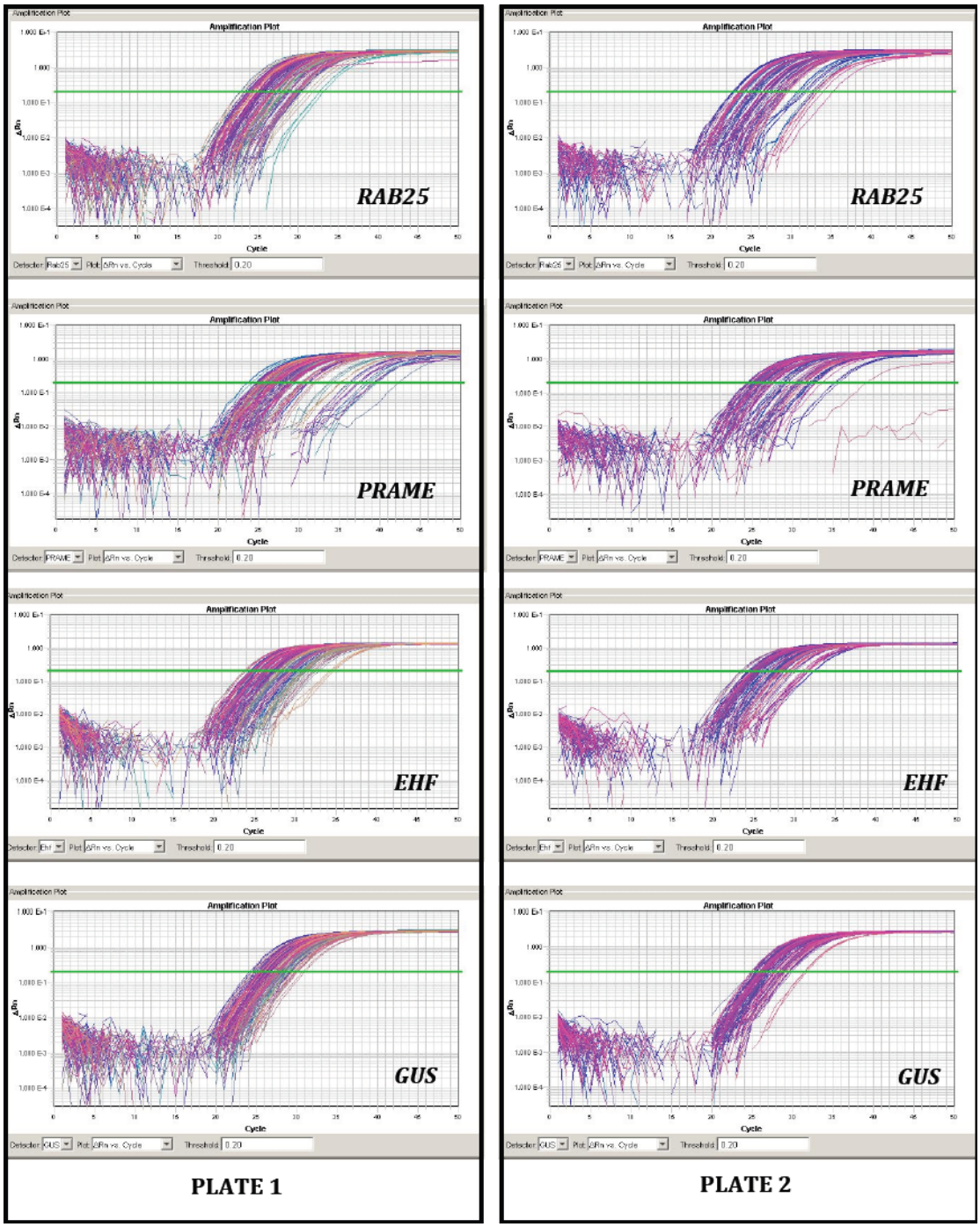


Figure 14: qRT-PCR plots for *RAB25, PRAME, EHF* and *GUS* mRNA expression in 98 OC/PPC effusions, cases run in triplicate.

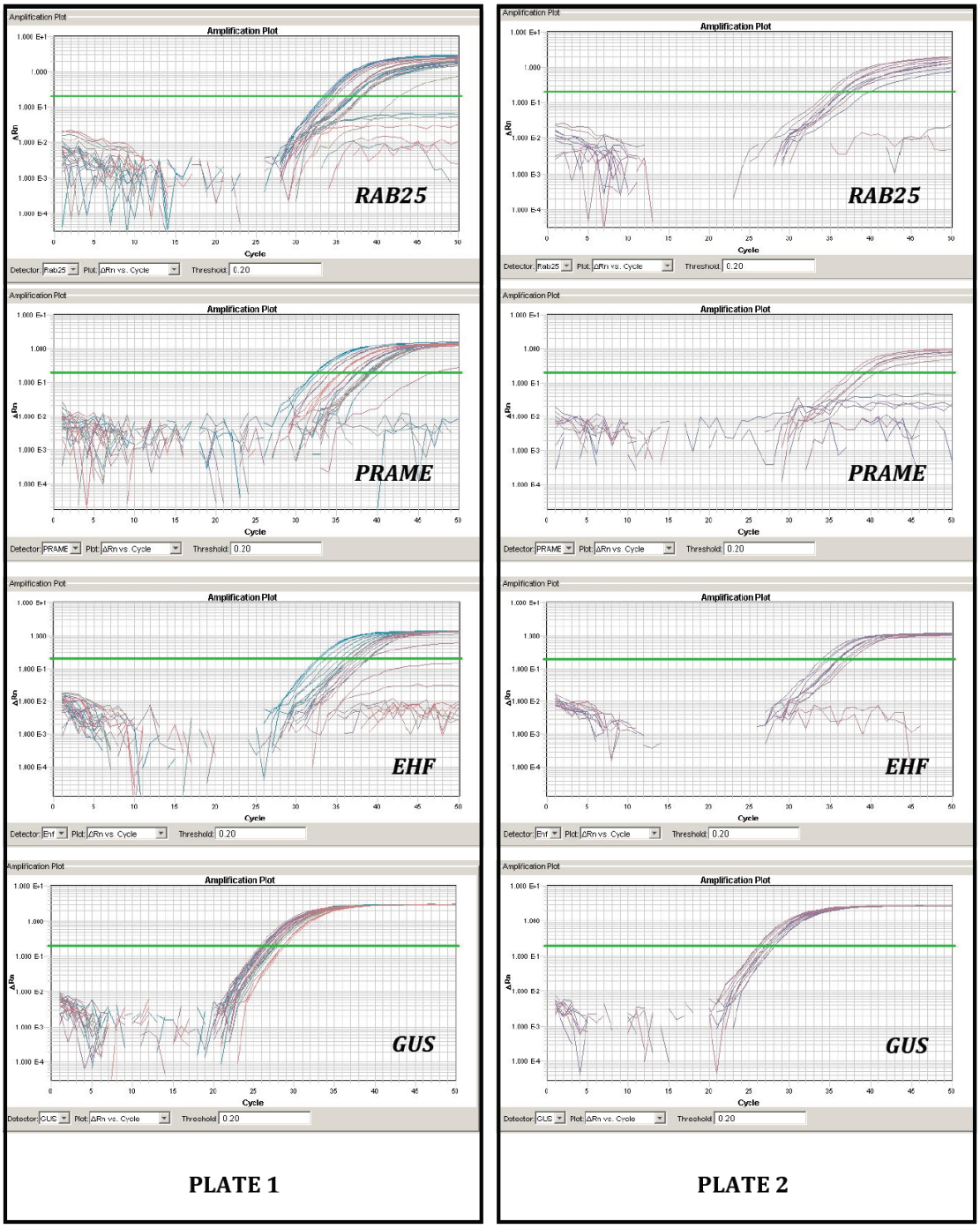


Figure 15: qRT-PCR plots for *RAB25, PRAME, EHF* and *GUS* mRNA expression in 14 MM effusions, cases run in triplicate.

Gene expression levels of *RAB25, PRAME* and *EHF* varied among the different tumor groups; primary OC (n=28), OC/PPC effusions (n=98) and MM effusions (N=14). The cycle threshold value was clearly lower for MM effusions compared to primary OC and OC/PPC effusions (Figure 13, 14 and 15). Final copy number values were established as a ratio with the level of the internal reference gene *GUS* in order to obtain comparable expression data shown in figure 16. *RAB25* copy numbers were as follows: primary OC range: 22-3386, median 763; OC/PPC effusions range: 1-3204, median 344, MM range: 0-1, median 0. *PRAME* copy numbers were as follows: primary OC range: 0-2894, median 499; OC/PPC effusions range: 0-2733, median 310, MM range: 0-7, median 0. *EHF* copy numbers were as follows: primary OC range: 74-21949, median 1535; OC/PPC effusions range: 3-4881, median 449, MM range: 0-5, median 0.5. Values of the reference gene *GUS* showed little variation among the samples within the different sample types; primary OC, OC/PPC effusions and MM effusions. Non-template controls (NTC) gave satisfactory results in all experiments.

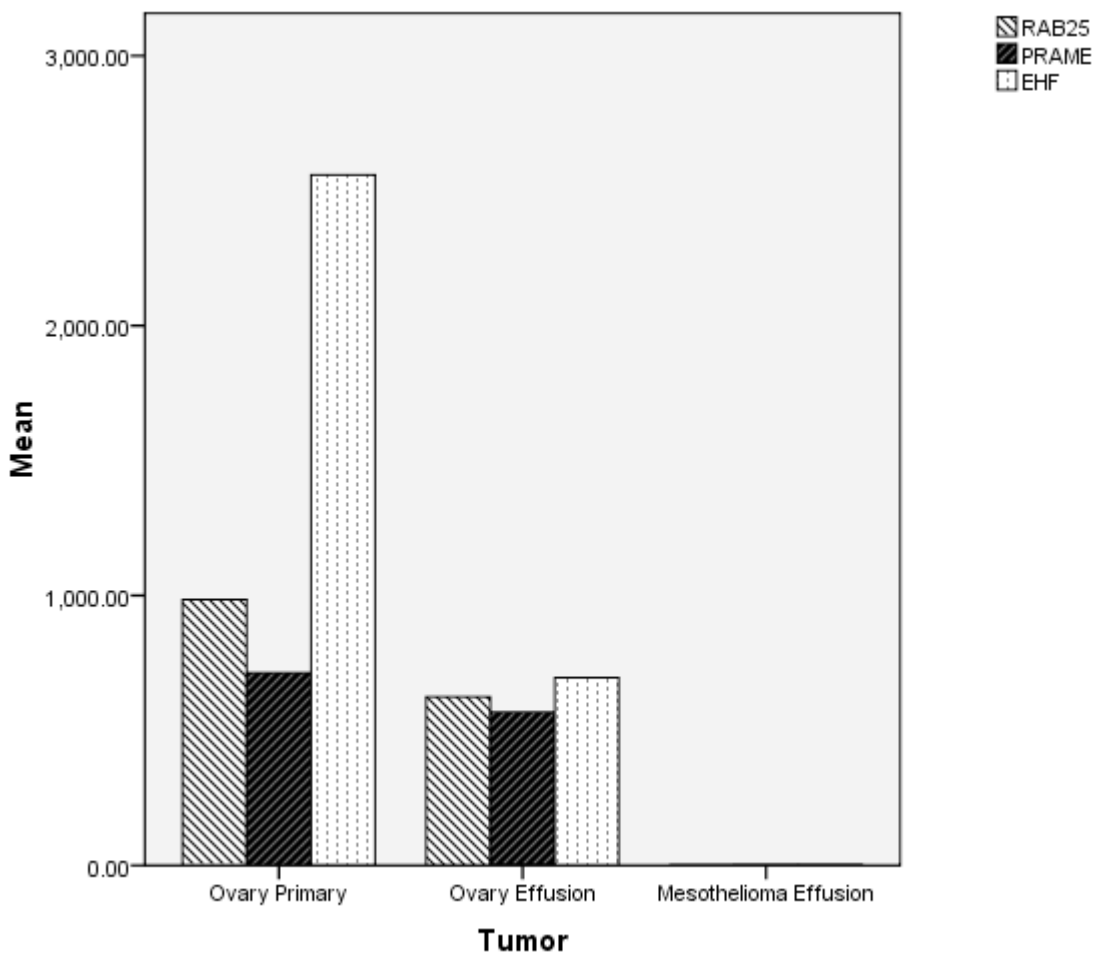


Figure 16: Mean gene expression level of *RAB25, PRAME* and *EHF* normalized against the internal reference gene *GUS* for the different tumor types; primary OC (n=28), OC/PPC effusions (n=98) and MM effusions (n=14).

4.4. Upregulation of all three genes; *RAB25, PRAME* and *EHF* in OC/PPC effusions compared to MM effusions

The microarray study published by Davidson et al. in 2006 showed that *RAB25, PRAME* and *EHF* are upregulated in OC/PPC effusions compared to MM effusions, with a 712, 114 and 120 fold change, respectively. The normalized expression data generated in this study validate these findings in a large sample material.

Considerable differences were observed in OC/PPC effusion samples (n=98) compared to MM effusion samples (n=14) with respect to copy number for all three genes, *RAB25, PRAME* and *EHF*. The results showed a significantly higher level of gene expression in the OC/PPC effusion samples of all three genes (*RAB25:* mean rank = 62.5 and 7.1 for OC/PPC and MM, respectively; *PRAME:* mean rank = 59.7 and 9.0 for OC/PPC and MM, respectively; *EHF:* mean rank = 59.5 and 5.8 for OC/PPC and MM respectively; $P<0.001$ for all three genes).

4.5. Differential gene expression levels of *EHF* between primary OC and OC/PPC effusions

As for primary OC and OC/PPC effusions, statistical analysis revealed that *EHF* was the only gene out of the three genes studied which had significantly higher expression levels in primary carcinomas compared to effusions (mean rank = 90.9 and 55.7 for primary OC and OC/PPC effusions, respectively; $P<0.001$). In addition one can infer a similar trend in the case of *RAB25* even though the statistics does show a significant difference (mean rank = 74.8 and 60.3 for primary OC and OC/PPC effusions, respectively; $P=0.062$).

4.6. Gene expression levels of *RAB25, PRAME* and *EHF* do not differentiate the OC/PPC effusions based on clinicopathologic parameters

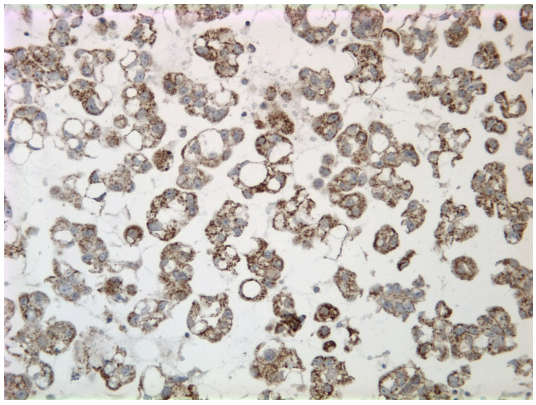
Clinical data were available for all patients with OC/PPC effusion samples. Thus, the expression level data for this group were used in statistical analysis of the clinicopathologic parameters. *RAB25, PRAME* and *EHF* mRNA was expressed in all 98 OC/PPC effusions, but expression levels were not related to any of the clinicopathologic parameters analyzed, i.e. effusion site, patient age, histological

grade, FIGO stage, residual disease volume, previous chemotherapy administration, response to chemotherapy at diagnosis and at first disease recurrence (P >0.05; data not shown).

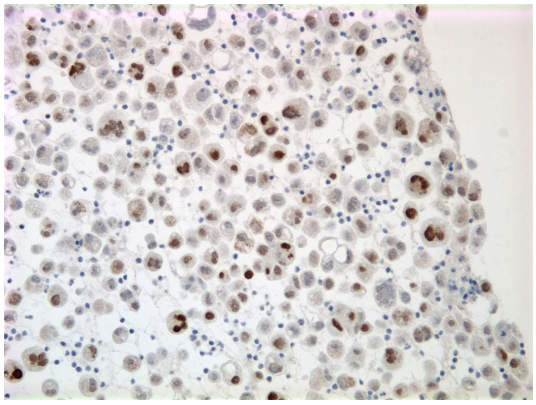
4.7. RAB25 protein expression level as a diagnostic marker

RAB25 protein expression was analyzed in 346 tumors, consisting of 209 OC/PPC effusions, 36 MM effusions and 101 solid OC. RAB25 was expressed in the cytoplasm of tumor cells in 189/209 (90%) OC/PPC effusions, with the following staining score: 0: 20; 1: 14; 2: 26; 3:46, 4: 103 (example of cytoplasmic staining in OC/PPC effusions is shown in figure 17 A). In MM effusions, RAB25 was expressed in 12/36 (33%) specimens, with the following staining score: 0: 24; 1: 3; 2: 2; 3: 6, 4: 1. Nuclear expression was observed in 33/209 OC/PPC effusions, but none of the MM cases (example of nuclear staining in OC/PPC effusions is shown in figure 17 B). Mann-Whitney U test was performed separately for cytoplasmic and nuclear staining. This test showed a significantly higher RAB25 protein in the OC/PPC effusions compared to MM effusions for both staining parameters, cytoplasmic and nuclear (cytoplasmic staining: mean rank = 135.88 and 48.24 for OC/PPC and MM respectively, nuclear staining: mean rank = 125.58 and 106.50 for OC/PPC and MM respectively; P<0.001 for cytoplasmic staining and P=0.011 for nuclear staining). Positive and negative controls gave satisfactory results in all experiments. RAB25 was expressed in the cytoplasm of tumor cells in 31/34 (91%) primary OC, with the following staining score: 0: 3; 1: 4; 2: 1; 3: 6, 4: 20 (example of cytoplasmic staining in primary OC is shown in figure 17 C). In solid metastases, RAB25 was expressed in 63/67 (94%) specimens, with the following staining score: 0: 4; 1: 14; 2: 7; 3: 13, 4: 29.

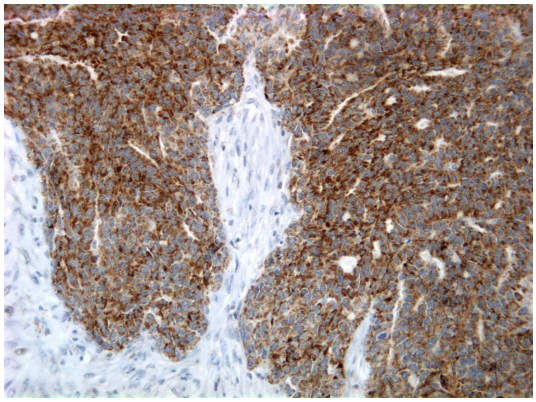
Wilcoxon Signed Ranks Analysis of the association between IHC results and anatomic site showed no significant differences in RAB25 cytoplasmic or nuclear staining between primary OC and solid metastases, or between effusions and solid primary or metastatic lesions ($p>0.05$; data not shown). However there was significantly higher nuclear RAB25 protein expression in histological grade 3 compared to grade 1-2 within the OC/PPC effusions (nuclear staining: mean rank = 98.87 and 86.23 for grade 3 and grade 1-2 respectively; $P=0.018$).



A)



B)



C)

Figure 17: RAB25 protein expression in: A) the cytoplasm of OC/PPC effusion cells, B) the nucleus of OC/PPC effusion cells, and C) the cytoplasm of primary OC cells.

4.8. Gene expression levels of *RAB25, PRAME* and *EHF*, and protein expression levels of RAB25 in relation to survival for patients with OC/PPC effusions

The follow-up period for the 93 OC/PPC patients with effusions analyzed using qRT-PCR ranged from 1 to 120 months (median, 25 months). PFS ranged from 0 to 66 months (median, 4 months), with 38 patients never achieving a disease-free period. At the last follow-up, 1 patient was alive with no evidence of disease, 4 patients were alive without disease, and 87 patients were dead of disease. One patient died of unlreated cause. In univariate survival analysis of the entire OC/PPC effusion cohort, no association was found between the expression levels of *RAB25, PRAME* and *EHF* (grouped as low or high based on median values) in relation to OS or PFS (P>0.05). However, in separate survival analysis of patients with prechemotherapy OC/PPC effusions, higher *EHF* expression (n=22) correlated with a shorter PFS of 3 months compared to 12 months for lower *EHF* expression (n=23) (P=0.01) (Figure 18). Clinical parameters that significantly correlated with PFS in this patient group were residual disease volume and response to chemotherapy for primary disease. Patients with a residual disease volume ≤1 cm (n= 13) had an estimated PFS of 19 months compared to 4 months for patients with residual disease volume > 1 cm (n=23) (P=0.003). Patients with complete response to chemotherapy at primary disease (n= 23) had an estimated PFS of 15 months compared to 0.2 months for patients with partial response, stable disease progression or allergic or adverse reaction to chemotherapy at primary disease (n=22) (P<0.001).

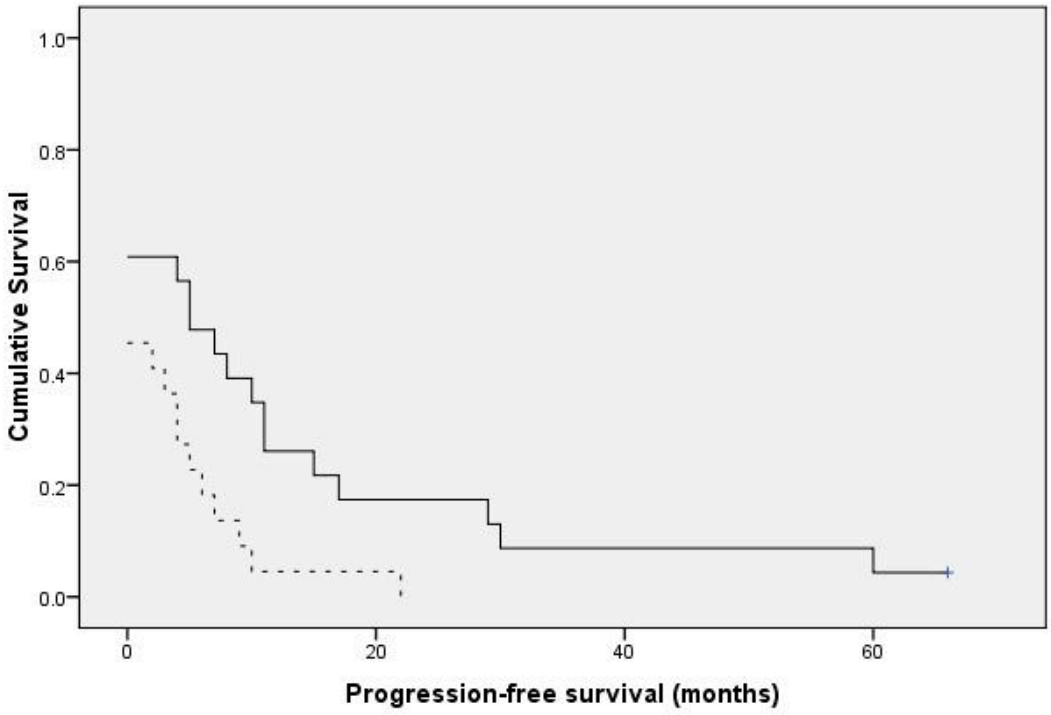


Figure 18: Kaplan-Meier survival curve showing correlation between *EHF* expression in OC/PPC effusions and PFS in the prechemotherapy cohort (45 patients).

Multivariate analysis was performed for the prechemotherapy OC/PPC effusions, and the parameters entered in to the Cox analysis of PFS were *EHF* expression, residual disease volume and response to chemotherapy at primary disease. All three parameters proved to be independent predictors of PFS (P=0.027, P=0.029 and P<0.000 for *EHF* expression, residual disease volume and response to chemotherapy at primary disease respectively).

Protein expression levels of RAB25 were unrelated to PFS or OS (P>0.05) when examining the OC/PPC effusions as one entity, and also when separated into prechemotherapy and postchemotherapy effusions.

5. DISCUSSION

Despite the recent improvements in the IHC panels used to obtain a correct diagnosis of tumors, there are still difficulties encountered in effusion cytology and surgical pathology. These difficulties are evident when distinguishing between intricate cases of serous OC/PPC and MM, and when determining the site of origin for metastatic adenocarcinoma (82, 137-141). As OC/PPC and MM present with similar clinical symptoms (especially within the peritoneal cavity), morphological resemblance and coexpression or lack of expression of several molecular markers, these two tumor types are especially difficult to discriminate from one another. Examination of surgical specimens has shown that podoplanin, calretinin, cytokeratin 5/6, WT1, and mesothelin are markers that are expressed in both OC/PPC and MM tumors, whilst both tumors are carcinoembryonic antigen negative (reviewed in ref 138 and 140). These findings have later been shown to apply to effusion cytology as well (73, 142).

Based on the cDNA microarray study conducted by Davidson et al. in 2006 (ref 133) where 189 genes were found to have differential expression between DMPM and OC/PPC specimens, this present study examined the mRNA expression of *RAB25, PRAME* and *EHF* in addition to RAB25 protein expression,

in a large sample material consisting of primary OC, OC/PPC effusions and MM effusions.

The first aim of this study was to develop and establish qRT-PCR assays for *RAB25* and *PRAME* that were to be used for examination of gene expression in our sample material. Several alternative primer combinations were tested for amplicon generation for each of the genes, and the most specific combinations were tested further with SYBR Green chemistry and synthetic oligonucleotides as standards for use by qRT-PCR. Gene specific assays that showed good differentiation within the standard curve were used further in combination with a target specific TaqMan probe. This final combination with the most specific primer set together with the corresponding TaqMan probe was tested together with the synthetic oligonucleotides used as standards, and the assay proved to be highly specific and show good differentiation within the range of 500,000 to 50 copies. This applied to both of the generated qRT-PCR assays; *RAB25* and *PRAME*, thus these assays were chosen to be used as part of the mRNA expression analysis for this study.

The second aim of this study was to measure the gene expression levels of *RAB25, PRAME* and *EHF* within the OC/PPC and MM effusions and by doing so validate the findings of the cDNA microarray study. The results generated from this study showed significantly higher expression levels of *RAB25, PRAME* and *EHF* mRNA in the OC/PPC effusions compared to the MM effusions. These results

comply perfectly with the previously established results of the cDNA microarray study where all three genes were found to have a higher expression in the OC/PPC effusions compared with the MM effusions (133).

The last two aims of this study were to analyze and compare the gene expression profile of the three genes in the entire sample material; primary OC, OC/PPC effusions and MM effusions. In addition, the potential differences in RAB25 protein expression between OC/PPC and MM samples were analyzed using IHC. Finally, the diagnostic and prognostic role of these biomarkers was evaluated. The gene expression levels of *RAB25, PRAME* and *EHF* were significantly different between OC/PPC effusions and MM effusions. Little to no expression of *RAB25, PRAME* and *EHF* was detected in the MM effusions, whilst the OC/PPC effusions all had distinct expression of the three genes. This clear cut difference in mRNA expression levels provides strong evidence of the qRT-PCR assays for *RAB25, PRAME* and *EHF* being able to distinguish between the two sample types, and as such these qRT-PCR assays might have a role in establishing a diagnosis in cases with equivocal effusion cytology.

Upregulation of *RAB25* mRNA expression has also been noted in other carcinomas. A study by He H et al. showed increased *RAB25* expression levels in 11 hepatocellular carcinomas and in 1 cholagiocarcinoma (143). A significant upregulation of *RAB25* has also been found in transitional cell carcinoma (TCC) of the bladder, and in invasive breast tumor cells (144-145). Results obtained in

our study clearly show *RAB25* mRNA expression to be significantly upregulated in OC samples and as such concurs with studies carried out by other investigators that have shown *RAB25* to be overexpressed in both ovarian and breast cancer (103).

Increased gene expression of *PRAME* has been observed in melanoma, where 88% of primary lesions and 95% of metastases express this molecule, as well as in non-small cell lung carcinoma, breast carcinoma, renal cell carcinoma, head and neck carcinomas, Hodgkin's lymphoma, sarcomas, Wilms' tumor, medulloblastomas and in the acute and chronic phases of both myeloid and lymphocytic leukemia (109). The significant increase in *PRAME* mRNA expression in OC effusion samples thus adds to the list of cancers in which *PRAME* expression is upregulated. The association between upregulation of *PRAME* expression and carcinomas provides an indication of *PRAME* playing a role in tumorigenesis.

Elevated expression levels of *EHF* mRNA have previously been detected in breast and colorectal cancer (146-148). With the exception of the previous cDNA microarray study by our group, the upregulation of *EHF* gene expression has not been linked to OC in any other published study to date. Thus the findings of this study provide provided further insight into the role of *EHF* in carcinogenesis.

In the comparison of primary OC and OC/PPC effusions, the results showed a significant difference in the gene expression levels of *EHF*, with primary carcinomas having higher expression than effusions. A similar trend was noted for *RAB25*. From these results one can infer that there is decreased expression of *EHF* and *RAB25* as the tumor progresses and metastasizes from its solid form to effusions. The altered expression profile of tumor cells in effusions can result from a change in the microenvironment. When the tumor cells are part of solid organs they are complemented by various cell populations such as stromal myofibroblasts, endothelial cells and leukocytes. Within solid organs the tumor cells are able to induce leaky vessels and obtain nutrients and oxygen, in addition to gaining access to the circulation. When present in effusions, however, the tumor cells are in a hypoxic microenvironment with reduced access to nutrients, and no longer in interaction with stromal myofibroblasts and endothelial cells (92). The decreased expression levels of *EHF*, and to a lesser extent *RAB25*, in OC/PPC effusions compared to primary OC, might be a result of such a change in the microenvironment.

The diagnostic role of RAB25 was subsequently studied at the protein level. Similar to the mRNA expression levels of *RAB25,* protein expression of RAB25 was also significantly higher in OC/PPC effusions compared to MM, showing a good correlation between mRNA expression and protein expression. In addition our results also show that RAB25 protein has a significantly higher nuclear expression in grade 3 OC/PPC effusions, compared to grade 1-2. Our findings of RAB25 being present in the nucleus are highly interesting, as RABs are known to

be mostly cytosolic. However there are studies that show RAB25 related proteins to be located within the nucleus. One of these proteins are RBEL1B (Rab-like protein 1 B), and it is known to play an important part in breast tumorigenesis (149). Another nucleus-associated protein is RAN. The RAN protein resides on a branch of the RAB subfamily, and it is involved in nuclear import and export (150). The fact that protein expression of RAB25 is significantly higher in OC/PPC effusions compared to MM effusions, in addition to significantly higher expression in grade 3 compared to grade 1-2 OC/PCC effusions, makes it a candidate diagnostic marker to be used in the IHC panel for effusion cytology.

Previous studies carried out by Davidson et al. report on differences in expression and clinical significance of cancer-associated molecules between carcinoma cells in pre- and postchemotherapy effusions (92, 151). Thus, analysis of the relationship between *RAB25, PRAME* and *EHF* mRNA expression in OC/PPC effusions and response to chemotherapy at diagnosis and at first disease recurrence was conducted. The results did not show any difference in gene expression of any of the three genes when pre- and postchemotherapy specimens were compared, nor when comparing gene expression and chemotherapy response. This suggests that mRNA expression of *RAB25, PRAME* and *EHF* in OC/PPC effusions is unaffected by chemotherapy.

When examining the prognostic role of *RAB25, PRAME* and *EHF* mRNA expression in OC/PPC effusions, the results showed correlation between

elevated *EHF* mRNA expression and poor PFS in the prechemotherapy effusions. Complying with previously published studies (8, 16, 33-34), this prechemotherapy patient group also showed a significant variation of PFS in relation to the clinical parameter of residual disease volume with patients having a residual tumor of larger than 1 cm having a poor PFS. In addition the results showed that the prechemotherapy patient group with partial response, stable disease progression or allergic or adverse reaction to chemotherapy at primary disease had poor PFS. In Cox multivariate analysis in which these three parameters were entered, all, including *EHF* mRNA expression, were independent predictors of shorter PFS. This suggests that EHF is a novel prognostic marker in OC.

6. CONCLUSION

In conclusion, qRT-PCR analysis confirmed the higher expression of *RAB25, PRAME* and *EHF* mRNA in OC/PPC effusions compared to MM effusions observed in the previous cDNA array analysis, supporting the use of *RAB25, PRAME* and *EHF* as novel diagnostic markers in OC. Similarly, the pronounced differences in RAB25 protein expression between OC/PPC effusions and MM effusions using IHC suggest that this protein may be a novel marker to be included in the diagnostic panel of serosal tumors. The qRT-PCR analysis also revealed a significantly higher expression of *EHF* mRNA in primary OC compared to OC/PPC effusions, and suggested a similar trend for the expression of *RAB25* mRNA, suggesting that expression of these molecules is altered along tumor progression in this cancer. The significant association between higher expression of *EHF*

mRNA in pre-chemotherapy OC/PPC effusions and poor survival suggest a potential role as a novel prognostic marker in OC.

7. FUTURE PERSPECTIVES

Even though it is well known that the different OC subtypes; serous, clear cell, endometrioid and mucinous, have highly variable molecular profiles and somewhat different clinical course, there is still a trend for investigators to examine variation in biomarkers on sample material that is not subtype-specific. The tumors analyzed used in this study were predominantly of the serous subtype. Studies that have used a subtype specific approach when examining biomarker expression profiles have provided evidence supporting the hypothesis that OC subtypes should be considered to be different diseases (152). Based on this study of serous OC, it could be interesting to use the assays developed in this project and investigate the expression level patterns of *RAB25, PRAME* and *EHF* in a sample material consisting of the remaining OC subtypes to see if our findings are OC subtype specific. An additional extension of this study would be to analyze solid metastases for expression of these genes in order to obtain a full picture with respect to their dynamics and clinical role in OC tumor progression.

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Appendices:

Appendix I Reagents, equipment and software

Appendix II Sequences and real-time RT-PCR products

Appendix I

Reagents, equipment and software

The following reagents, equipment and software were used in this thesis.

Reagents (product, catalogue number and manufacturer)

Agarose, SeaKem®, 50004, Cambrex

Antibody Diluent, S0809, DakoCytomation

DAB+, K3468, DakoCytomation

DEPC (dietyl pyrokarbonat) treated water, 10813-012, Invitrogen

DNA-free Kit, AM1906, Applied Biosystems

DMSO (dimethyl sulfoxide), D8418, Sigma

dNTP set high conc. 100mM 4x125umol, 69689-2, VWR

EnVision+ System, K4011, DakoCytomation

GUS control gene standard, CGRS-03, Ipsogen

Haematoxylin, S3301, DakoCytomation

Hydrogen peroxide 0.03%, S2001, DakoCytomation

PBS (phosphate buffered saline), 17-516F, Medprobe

Phusion Hot Start enzyme, F-540L, Sigma

Platinum qPCR Supermix UDG with Rox, 11743-500, Invitrogen

Power SYBR Green PCR mastermix, 4367659, Applied Biosystems

QIAamp RNeasy Mini Kit, 74104, Qiagen

Random primers, 48190-011, Invitrogen

RNase OUT, 10777-019, Invitrogen

RNeasy Fibrous Tissue Mini Kit, 74704, Qiagen

SuperscriptTM III Reverse Transcriptase, 18080-085, Invitrogen

SybrSafe, S33102, Invitrogen

Equipment (product, catalogue number/model and manufacturer)

ART filter tips 1000, 133.605-1000, VWR

ART filter tips 200, 133.602-201, VWR

ART filter tips 20P, 133.601-22, VWR

ART filter tips 10 Reach 133.600-11, VWR

BioRad PowerPack 300, BioRad

Biomek AP96 P250 Tips Sterile Barrier, 717253, Nerlien Mezanski

Biomek AP96 P20 Tips Sterile Barrier, 717256, Nerlien Mezanski

Biomek 3000, 986120, BeckmanCoulter

Eppendorf centrifuge 5804, 5804 000.013, Eppendorf

Finnpipettes, Thermo Labsystems

GeneFlash, Syngene Bioimaging

Gilson pipettes, Gilson Inc.

Petridish, 1066, Heger AS

QIAcube, 9001293, Qiagen

Thermo Fast 96 non-skirted plate, ADVAAB-0600, VWR

TissueRuptor, 9001272, Qiagen

7900HT Sequence Detection System, Applied Biosystems

96-well GeneAmp PCR system 2720, Applied Biosystems

BioPhotometer 6131, Eppendorf

Software

7900HD Sequence Detection System, version 2.3, Applied Biosystems

Biomek 3000 software, BeckmanCoulter

Freeware

Ensembl Genome Browser
(http://www.ensembl.org/index.html)

Finnzymes Reagents- Tm Calculator
(https://www.finnzymes.fi/tm_determination.html)

NCBI; National Center for Biotechnology Information
(http://www.ncbi.nlm.nih.gov/)

NetPrimer software, PREMIER Biosoft
(http://www.premierbiosoft.com/netprimer/index.html)

SDSC Biology Workbench
(http://workbench.sdsc.edu/)

Appendix III:

RAB25 sequence, NM_020387.2 (1101 bp)

Real-time PCR product: 229-325

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   1 CTCTGCTTCC TTACAGCACC CCCACCTGCC AGAGCTGATC CTCCCTAGGC CCTGCCTAAC
  61 CTTGAGTTGG CCCCCAATCC CTCTGGCTGC AGAAGTCCCC TTACCCCCAA TGAGAGGAGG
 121 GGCAGGACCA GATCTTTTGA GAGCTGAGGG TTGAGGGCAT TGAGCCAACA CACAGATTTG
 181 TCGCCTCTGT CCCCGAAGAC ACCTGCACCC TCCATGCGGA GCCAAGATGG GGAATGGAAC
 241 TGAGGAAGAT TATAACTTTG TCTTCAAGGT GGTGCTGATC GGCGAATCAG GTGTGGGGAA
 301 GACCAATCTA CTCTCCCGAT TCACGCGCAA TGAGTTCAGC CACGACAGCC GCACCACCAT
 361 CGGGGTTGAG TTCTCCACCC GCACTGTGAT GTTGGGCACC GCTGCTGTCA AGGCTCAGAT
 421 CTGGGACACA GCTGGCCTGG AGCGGTACCG AGCCATCACC TCGGCGTACT ATCGTGGTGC
 481 AGTGGGGGCC CTCCTGGTGT TTGACCTAAC CAAGCACCAG ACCTATGCTG TGGTGGAGCG
 541 ATGGCTGAAG GAGCTCTATG ACCATGCTGA AGCCACGATC GTCGTCATGC TCGTGGGTAA
 601 CAAAAGTGAC CTCAGCCAGG CCCGGGAAGT GCCCACTGAG GAGGCCCGAA TGTTCGCTGA
 661 AAACAATGGA CTGCTCTTCC TGGAGACCTC AGCCCTGGAC TCTACCAATG TTGAGCTAGC
 721 CTTTGAGACT GTCCTGAAAG AAATCTTTGC GAAGGTGTCC AAGCAGAGAC AGAACAGCAT
 781 CCGGACCAAT GCCATCACTC TGGGCAGTGC CCAGGCTGGA CAGGAGCCTG GCCCTGGGGA
 841 GAAGAGGGCC TGTTGCATCA GCCTCTGACC TTGGCCAGCA CCACCTGCCC CCACTGGCTT
 901 TTTGGTGCCC CTTGTCCCCA CTTCAGCCCC AGGACCTTTC CTTGCCCTTT GGTTCCAGAT
 961 ATCAGACTGT TCCCTGTTCA CAGCACCCTC AGGGTCTTAA GGTCTTCATG CCCTATCACA
1021 AATACCTCTT TTATCTGTCC ACCCCTCACA GACTAGGACC CTCAAATAAA GCTGTTTTAT
1081 ATCAAAAAAA AAAAAAAAAA A
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PRAME sequence, NM_206953.1 (2776 bp)

Real-time PCR product: 1800-1883

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   1 CGAGTTCCGG CGAGGCTTCA GGGTACAGCT CCCCCGCAGC CAGAAGCCGG GCCTGCAGCG

  61 CCTCAGCACC GCTCCGGGAC ACCCCACCCG CTTCCCAGGC GTGACCTGTC AACAGCAACT

 121 TCGCGGTGTG GTGAACTCTC TGAGGAAAAA CGTAAGTTCG AGCCCTGATT CCTCCGCTTC

 181 CCCGCAGGGT GACCTTGGGC TTGTGCCCCC GGCACCACCC CTGTCCCGGG TCCCTGTTTT

 241 CTCTCTGGAA ATGGGTTGAA GACCAAAGAA AATAATGTGC GCCACTTGGG TCACCCCGGG

 301 CCGCCTGCCC CGGAAAATTG GCCCCAGTTG AGGAGTTGTG GCTGTAAGGA TGCCTTGAAC

 361 CGAGGCGGCG GTGCTCGTGG TTGGAGCTCT CCAGGGTGGG TGCGCATTTG TAATGCGGTG

 421 GATGCTCTGG GACTCGGCCC CTCTGAAGGT GCTGGGGGTT GGGGACGGCC CAGGCAGTGG

 481 CGTAGGCGTC CTAGGAAGGC GGGAGCAGAG GCAGAAATGT CGCTGCAAGA CCGTAGTCAG

 541 GGTCCTTGAC CACAGGGGTC ACTTGTGACC AACCACATGG TCTGTTGTTC CTCCTGCCCC

 601 CTGGTTCAGC CCAGGAAACA CTGGTGCTCA GGTTTGGAGC CAGAGATTTG CACTGAAAGG

 661 GCGGGATTGA GTCGCCAGTT GTCAGTTTCC TCAGCAGTAT TTGCGGAGGT TTTCACAGGA

 721 GGCCGTTGCT TCGTAAATAT TATACATGTA TTCTTCTTTT TGGAGCATTT TGATTATTAC

 781 TCTCAGACGT GCGTGGCAAC AAGTGACTGA GACCTAGAAA TCCAAGCGTT GGAGGTCCTG

 841 AGGCCAGCCT AAGTCGCTTC AAAATGGAAC GAAGGCGTTT GNGGGGTTCC ATTCAGAGCC

 901 GATACATCAG CATGAGTGTG TGGACAAGCC CACGGAGACT TGTGGAGCTG GCAGGGCAGA

 961 GCCTGCTGAA GGATGAGGCC CTGGCCATTG CCGCCCTGGA GTTGCTGCCC AGGGAGCTCT

1021 TCCCGCCACT CTTCATGGCA GCCTTTGACG GGAGACACAG CCAGACCCTG AAGGCAATGG

1081 TGCAGGCCTG GCCCTTCACC TGCCTCCCTC TGGGAGTGCT GATGAAGGGA CAACATCTTC

1141 ACCTGGAGAC CTTCAAAGCT GTGCTTGATG GACTTGATGT GCTCCTTGCC CAGGAGGTTC

1201 GCCCCAGGAG GTGGAAACTT CAAGTGCTGG ATTTACGGAA GAACTCTCAT CAGGACTTCT
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1261 GGACTGTATG GTCTGGAAAC AGGGCCAGTC TGTACTCATT TCCAGAGCCA GAAGCAGCTC

1321 AGCCCATGAC AAAGAAGCGA AAAGTAGATG GTTTGAGCAC AGAGGCAGAG CAGCCCTTCA

1381 TTCCAGTAGA GGTGCTCGTA GACCTGTTCC TCAAGGAAGG TGCCTGTGAT GAATTGTTCT

1441 CCTACCTCAT TGAGAAAGTG AAGCGAAAGA AAAATGTACT ACGCCTGTGC TGTAAGAAGC

1501 TGAAGATTTT TGCAATGCCC ATGCAGGATA TCAAGATGAT CCTGAAAATG GTGCAGCTGG

1561 ACTCTATTGA AGATTTGGAA GTGACTTGTA CCTGGAAGCT ACCCACCTTG GCGAAATTTT

1621 CTCCTTACCT GGGCCAGATG ATTAATCTGC GTAGACTCCT CCTCTCCCAC ATCCATGCAT

1681 CTTCCTACAT TTCCCCGGAG AAGGAAGAGC AGTATATCGC CCAGTTCACC TCTCAGTTCC

1741 TCAGTCTGCA GTGCCTGCAG GCTCTCTATG TGGACTCTTT ATTTTTCCTT AGAGGCCGCC

1801 TGGATCAGTT GCTCAGGCAC GTGATGAACC CCTTGGAAAC CCTCTCAATA ACTAACTGCC

1861 GGCTTTCGGA AGGGGATGTG ATGCATCTGT CCCAGAGTCC CAGCGTCAGT CAGCTAAGTG

1921 TCCTGAGTCT AAGTGGGGTC ATGCTGACCG ATGTAAGTCC CGAGCCCCTC CAAGCTCTGC

1981 TGGAGAGAGC CTCTGCCACC CTCCAGGACC TGGTCTTTGA TGAGTGTGGG ATCACGGATG

2041 ATCAGCTCCT TGCCCTCCTG CCTTCCCTGA GCCACTGCTC CCAGCTTACN ACCTTAAGCT

2101 TCTACGGGAA TTCCATCTCC ATATCTGCCN TGCAGAGTCT CCTGCAGCAC CTCATCGGGC

2161 TGAGCAATCT GACCCACGTG CTGTATCCTG TCCCCCTGGA GAGTTATGAG GACATCCATG

2221 GTACCCTCCA CCTGGAGAGG CTTGCCTATC TGCATGCCAG GCTCAGGGAG TTGCTGTGTG

2281 AGTTGGGGCG GCCCAGCATG GTCTGGCTTA GTGCCAACCC CTGTCCTCAC TGTGGGGACA

2341 GAACCTTCTA TGACCCGGAG CCCATCCTGT GCCCCTGTTT CATGCCTAAC TAGCTGGGTG

2401 CACATATCAA ATGCTTCATT CTGCATACTT GGACACTAAA GCCAGGATGT GCATGCATCT

2461 TGAAGCAACA AAGCAGCCAC AGTTTCAGAC AAATGTTCAG TGTGAGTGAG GAAAACATGT

2521 TCAGTGAGGA AAAAACATTC AGACAAATGT TCAGTGAGGA AAAAAAGGGG AAGTTGGGGA

2581 TAGGCAGATG TTGACTTGAG GAGTTAATGT GATCTTTGGG GAGATACATC TTATAGAGTT

2641 AGAAATAGAA TCTGAATTTC TAAAGGGAGA TTCTGGCTTG GGAAGTACAT GTAGGAGTTA

2701 ATCCCTGTGT AGACTGTTGT AAAGAAACTG TTGAAAATAA AGAGAAGCAA TGTGAAGCAA

2761 AAAAAAAAAA AAAAAA

EHF sequence, NM_012153.3 (3617 bp)

Real-time PCR product: 83- 171

```
   1 ACCCGTGGTG CCCCATCCCT ATAGGAGCTG GTGAGATTGC AGCCTGCTGC CTCCCCTCCA

  61 TCAGCCACAG CTATTGGATT TCCCACCCAG AATCTTTAGG TAAATGAGAT CATGATTCTG

 121 GAAGGAGGTG GTGTAATGAA TCTCAACCCC GGCAACAACC TCCTTCACCA GCCGCCAGCC

 181 TGGACAGACA GCTACTCCAC GTGCAATGTT TCCAGTGGGT TTTTTGGAGG CCAGTGGCAT

 241 GAAATTCATC CTCAGTACTG GACCAAGTAC CAGGTGTGGG AGTGGCTCCA GCACCTCCTG

 301 GACACCAACC AGCTGGATGC CAATTGTATC CCTTTCCAAG AGTTCGACAT CAACGGCGAG

 361 CACCTYTGCA GCATGAGTTT GCAGGAGTTC ACCCGGGCGG CAGGGACGGC GGGGCAGCTC

 421 CTCTACAGCA ACTTGCAGCA TCTGAAGTGG AACGGCCAGT GCAGTAGTGA CCTGTTCCAG

 481 TCCACACACA ATGTCATTGT CAAGACTGAA CAAACTGAGC CTTCCATCAT GAACACCTGG

 541 AAAGACGAGA ACTATTTATA TGACACCAAC TATGGTAGCA CAGTAGATTT GTTGGACAGC

 601 AAAACTTTCT GCCGGGCTCA GATCTCCATG ACAACCACCA GTCACCTTCC TGTTGCAGAG

 661 TCACCTGATA TGAAAAAGGA GCAAGACCCC CCTGCCAAGT GCCACACCAA AAAGCACAAC

 721 CCGAGAGGGA CTCACTTATG GGAATTCATC CGCGACATCC TCTTGAACCC AGACAAGAAC

 781 CCAGGATTAA TAAAATGGGA AGACCGATCT GAGGGCGTCT TCAGGTTCTT GAAATCAGAG

 841 GCAGTGGCTC AGCTATGGGG TAAAAAGAAG AACAACAGCA GCATGACCTA TGAAAAGCTC

 901 AGCCGAGCTA TGAGATATTA CTACAAAAGA GAAATTCTGG AGCGTGTGGA TGGACGAAGA

 961 CTGGTATATA AATTTGGGAA GAATGCCCGA GGATGGAGAG AAAATGAAAA CTGAAGCTGC

1021 CAATACTTTG GACACAAACC AAAACACACA CCAAATAATC AGAAACAAAG AACTCCTGGA

1081 CGTAAATATT TCAAAGACTA CTTTTCTCTG ATATTTATGT ACCATGAGGG GAACAAGAAA

1141 CTACTTCTAA CGGGAAGAAG AAACACTACA GTCGATTAAA AAAATTATTT TGTTACTTCG

1201 AAGTATGTCC TATATGGGGA AAAAACGTAC ACAGTTTTCT GTGAAATATG ATGCTGTATG
```

1261 TGGTTGTGAT TTTTTTTCAC CTCTATTGTG AATTCTTTTT CACTGCAAGA GTAACAGGAT

1321 TTGTAGCCTT GTGCTTCTTG CTAAGAGAAA GAAAAACAAA ATCAGAGGGC ATTAAATGTT

1381 TTGTATGTGA CATGATTTAG AAAAAGGTGA TGCATCCTCC TCACATAAGC ATCCATATGG

1441 CTTCGTCAAG GGAGGTGAAC ATTGTTGCTG AGTTAAATTC CAGGGTCTCA GATGGTTAGG

1501 ACAAAGTGGA TGGATGCCGG GAAGTTTAAC CTGAGCCTTA GGATCCAATG AGTGGAGAAT

1561 GGGGACTTCC AAAACCCAAG GTTGGCTATA ATCTCTGCAT AACCACATGA CTTGGAATGC

1621 TTAAATCAGC AAGAAGAATA ATGGTGGGGT CTTTATACTC ATTCAGGAAT GGTTTATCTG

1681 ATGCCAGGGC TGTCTTCCTT TCTCCCCTTT GGATGGTTGG TGAAATACTT TAATTGCCCT

1741 GTCTGCTCAC TTCTAGCTAT TTAAGAGAGA ACCCAGCTTG GTTCTTTTTT GCTCCAAGTG

1801 CTTAAAAATA AGTTGGAAAA AGGAGACGGT GGTGTGGAAA TGGCTGAAGA GTTTGCTCTT

1861 GTATCCCTAT AGTCCAAGGT TTCTCAATCT GCACAATTGA CATTTTTGGC CGGAGTGTTC

1921 TTTGTGGTGA GGGCTTTCCT GTGCATTGTA AGATGTTCAG CAGTATCCAC TCATGGTCTC

1981 TAACCACTTG ACACCAGAAA CCCCCCAGCT GTGATAACGC AAAATGTCTC TAGACATCAC

2041 CAAATGTTCC CTGGGGGTGG CAAATTTGCC CTTGATTGAG AACCACCAGT TTAGCTAGTC

2101 AATATGAGGA TGGTGGTTTA TTCTCAGAAG AAAAAGATAT GTAAGGTCTT TTAGCTCCTT

2161 AGAGTGAAGC AAAAGCAAGA CTTCAACCTC AACCTATCTT TATGTTTTAA ATGTTAGGGA

2221 CAATAAGTTG AAATAGCTAG AGGAGCTTCT TTTCAGAACC CCAGATGAGA GCCAATGTCA

2281 GATAAAGTAA GCATAGTAAT GTAGCAGGAA CTACAATAGA AGACATTTTC ACTGGAATTA

2341 CAAAGCAGAA TTAAAATTAT ATTGTAGAAG GAAACACCAA GAAAAGAATT TCCAGGGAAA

2401 ATCCTCTTTG CAGGTATTAA TTCTTATAAT TTTTTGTCTT TTGGATTATC TGTTTACTGT

2461 CTCATCTGAA CTGATCCCAG GTGAACGGTT TATTGCCTAG ATTTGTACTC AGAGGAATTT

2521 TTTTTGTTTT GTTTTGTCTT TTAAGAAAGG AAAGAAAGGA TGAAAAAAAT AAACAGAAAA

2581 CTCAGCTCAG GCACAATTGT CACCAAGGAG TTAAAAGCTT CTTCTTCAAT AGAGGAATTG

2641 TTCTGGGGGT CCTGGAGACT TACCATTGAG CCATGCAATC TGGGAAGCAC AGGAATAAGT

2701 AGACACTTTG AAAATGGATT TGAATGTTCT CATCCCTTTT GCAGCTTTTC TTTTTGGCTC

2761 TCTCATGTCC TTGGCTTGCT CCTCTATTCT ACCTCTCTTT CTCCAGCAAT AATATGCAAA

2821 TGAAGACATG TATCCATAAG AAGGAGTGCT CTTCATCAAC TAATAGAGCA CCTACCACAG

2881 TGTCATACCT GGTAGAGGTG AGCAATTCAT ATTCAAAGGT TGCAAAGTGT TTGTAATATA

2941 TTCATGAGGC TGGAAGTAAG AAGAATTAAA AATTTGTCCT AATTACAATG AGAACCATTC

3001 TAGGTAGTGA TCTTGGAGCA CACATGAATA ACTTTCTGAA GGTGCAACCA AATCCATTTT

3061 TATTTCTGCC TGGCTTGGTC ACCTCTGTAA AGGTTTAACT TAGTGTTGTC AAGTAACAGT

3121 TACTGAAAGA GCTGAGAAAA AGAACAATGA ACAGCAACGA TCTTGACTGT GCAACTCAGA

3181 CATTCCTGCA GAAAAGACAT ATGTTGCTTT ACAAGAAGGC CAAAGAACTA TGGGGCCTTC

3241 CCAGCATTTG ACTGTTCATT GCATAGAATG AATTAAATAT CCAGTTACTT GAATGGGTAT

3301 AACGCATGAA TATTTGTGTG TCTGTGTGTG TGTCTGAGTT GTGTGATTTT ATTAGGGGCA

3361 TCTGCCAATT CTCTCACTGT GGTTCCTTCT CTGACTTTGC CTGTTCATCA TCTAAGGAGG

3421 CTAGATCCTT CGCTGACTTC ACCATTCCTC AAACCTGTAA GTTTCTCACT TCTTCCAAAT

3481 TGGCTTTGGC TCTTTCTGCA ACCTTTCCAT TCAAGAGCAA TCTTTGCTAA GGAGTAAGTG

3541 AATGTGAAGA GTACCAACTA CAACAATTCT ACAGATAATT AGTGGATTGT GTTGTTTGTT

3601 GAGAGTGAAG GTTTCTT